PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/705, C12N 9/74, C07K 16/28, C12N 1/21, 1/19, 5/10, C12Q 1/00, G01N 33/68, A61K 38/48, 38/17

A2

(11) International Publication Number: WO 99/43809

(43) International Publication Date: 2 September 1999 (02.09.99)

(21) International Application Number: PCT/US99/02983

(22) International Filing Date: 11 February 1999 (11.02.99)

(30) Priority Data:
09/032,397 27 February 1998 (27.02.98) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US).

(72) Inventors: COUGHLIN, Shaun, R.; 2 Turtle Rock Court, Tiburon, CA 94920 (US). KAHN, Mark; 1337 Carlos Avenue, Burlingame, CA 94010 (US).

(74) Agent: DEVORE, Dianna, L.; Bozicevic, Field & Francis LLP, Suite 200, 285 Hamilton Avenue, Palo Alto, CA 94301 (US).

(81) Designated States: AU, CA, JP, KR, NO, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PROTEASE-ACTIVATED RECEPTOR 4 AND USES THEREOF

(57) Abstract

Disclosed are cDNAs and genomic DNAs encoding protease-activated receptor 4 (PAR4) from mouse and human, and the recombinant polypeptides expressed from such cDNAs. The recombinant receptor polypeptides, receptor fragments and analogs expressed on the surface of cells are used in methods of screening candidate compounds for their ability to act as agonists or antagonists to the effects of interaction between thrombin and PAR4. Agonists are used as therapeutics to treat wounds, promote clotting, and as reagents to activate platelets in diagnostic tests. Antagonists are used as therapeutics to control blood coagulation, treat heart attack and stroke, and block inflammatory and proliferative responses to injury as occur in normal wound healing and variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and glomerulosclerosis. Antibodies specific for a protease-activated receptor 4 (or receptor fragment or analog) and their use as a therapeutic are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

PROTEASE-ACTIVATED RECEPTOR 4 AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to nucleic acids, their encoded protease-activated receptor 4 proteins, and screening assays for agonists and antagonists of the protease activated receptor 4 proteins.

BACKGROUND OF THE INVENTION

Thrombin, a coagulation protease generated at sites of vascular injury, activates platelets, leukocytes, and mesenchymal cells (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Activation of platelets by thrombin is thought to be critical for hemostasis and thrombosis. In animal models, thrombin inhibitors block platelet-dependent thrombosis, which is the cause of most heart attacks and strokes in humans. Available data in humans suggests that thrombosis in arteries can be blocked by inhibitors of platelet function and by thrombin inhibitors. Thus it is likely that thrombin's actions on platelets contribute to the formation of clots that cause heart attack and stroke. Thrombin's other actions on vascular endothelial cells and smooth muscle cells, leukocytes, and fibroblasts may mediate inflammatory and proliferative responses to injury, as occur in normal wound healing and a variety of diseases (atherosclerosis, restenosis, pulmonary inflammation (ARDS), glomerulosclerosis, etc.). A thorough understanding of how thrombin activates cells is an important goal.

A receptor that mediates thrombin signaling has been previously identified (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068; USP 5,256,766). This receptor revealed a novel proteolytic mechanism of activation and is referred to as PAR1 (protease-activated receptor 1). PAR1 is activated by the binding of thrombin to and cleavage of PAR1's amino terminal exodomain at a specific site. Receptor cleavage unmasks a new amino terminus, which then functions as a tethered peptide ligand by binding intramolecularly to the body of the receptor to effect transmembrane signaling (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Synthetic peptides that mimic this tethered ligand domain function as PAR1 agonists and activate it independent of thrombin and receptor cleavage (Vu, T.-K.H. et al. (1991) Cell 64:1057-1068).

To identify which of thrombin's known cellular actions are mediated by PAR1, a PAR1 knockout mouse was recently generated (Connolly, A. et al. (1996) Nature 381:516-

519). Analysis of mice in which both alleles of the PAR1 gene were disrupted provided definitive evidence for a second platelet thrombin receptor and for tissue specific roles of distinct thrombin receptors. Specifically, in mice, PAR1 is not important for platelet responses but is critical for fibroblast responses.

Since the identification of PAR1, two other protease-activated receptors have been cloned. A second protease-activated receptor (PAR2) was cloned during a search for relatives of the Substance K receptor (Nystedt, S., et al. (1994) PNAS USA, 91:9208-9212). The physiological activator of PAR2 remains unknown; it is not activated by thrombin. A third protease-activated receptor (PAR3) has also been cloned, but available data suggests that this receptor is involved in thrombin-mediated platelet responses in mice but not in humans.

There is a need for a better understanding of thrombin-mediated platelet activation. There is also a need for the identification and characterization of factors involved in platelet-mediated pathologies, such as platelet-dependent arterial thrombosis. The understanding of the mechanisms of such offer new mechanisms for treating associated pathologies.

SUMMARY OF THE INVENTION

Protease-activated receptor 4 (PAR4) is disclosed. PAR4 is useful in assaying libraries of compounds for their activity as thrombin agonists and antagonists. DNA encoding PAR4 is also disclosed as is its insertion into a functional expression vector, DNA expressed in a cell line, and the use of the DNA expression product in an assay to identify compounds as agonists or antagonists of thrombin's effect on PAR4.

The invention comprises substantially pure DNA (cDNA or genomic DNA) encoding a protease-activated receptor 4 (PAR4) from vertebrate tissues (SEQ ID NO:1, SEQ ID NO:3, AND SEQ ID NO:4) and degenerate sequences thereof; substantially pure protease-activated receptor 4 polypeptides encoded thereby; as well as amino acid sequences substantially identical to the amino acid sequences SEQ ID NO:2 and SEQ ID NO:5 from mouse and human, respectively. The invention also features DNA sequences that hybridize under stringent conditions to DNA encoding PAR4, or to DNA complementary to DNA encoding PAR4. Such DNA sequences are preferably at least 25 nucleotides in length, more preferably 50 nucleotides in length. The invention further comprises fragments of the PAR4 receptor which are activated by thrombin. Such fragments may have the same amino acid

sequence as SEQ ID NO:2 and SEQ ID NO:5 or be at least 80% identical to the amino acid sequences SEQ ID NO:2 and SEQ ID NO:5. Such fragments are preferably at least 10 amino acids in length, more preferably at least 30 amino acids in length.

In various embodiments, the DNA, receptor or receptor fragment is derived from a vertebrate animal, preferably, human or mouse. However, the gene can be chemically synthesized.

An object of the invention is to provide a nucleotide sequence encoding a novel receptor, preferably PAR4 and its functional equivalents.

Another object is to provide a cell line genetically engineered to express the nucleotide sequence encoding PAR4.

Another object is to provide an antibody which selectively binds the PAR 4 receptor.

Another object is to provide a method whereby a compound or library of compounds can be assayed for their ability to activate or block the receptor expressed by the nucleotide sequence.

An advantage of the present invention is that a novel thrombin receptor PAR4 is disclosed making it possible to identify novel thrombin agonists and antagonists which may not be identifiable via PAR1, PAR2, or PAR3 receptors.

A feature of the invention is that it makes it possible to obtain additional information regarding thrombin activation and the sequence of biochemical events initiated by such.

These and other objects, advantages and features of the present invention will become apparent to those skilled in the art upon reading the disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are the complete nucleotide sequences (SEQ ID NO:1) of the mouse protease-activated receptor 4 gene coding region cDNA.

Fig. 2 is the deduced amino acid sequence (SEQ ID NO:2) of the receptor encoded by the nucleotide sequence of Fig. 1. The amino acid sequence encoding mouse PAR4 contains 397 amino acids. The deduced amino acid sequence begins at nucleotides 1-3 (ATG = Met) and ends at nucleotides 1192-1194 (TGA = stop).

Fig. 3 is the nucleotide sequence of genomic mouse PAR4 (SEQ ID NO:3) demonstrating a small intron (250 bp) between the signal peptide and cleavage site of the receptor coding sequence. The intronic sequence is underlined.

Figs. 4A and 4B are the complete nucleotide sequences (SEQ ID NO:4) of the human protease-activated receptor 4 gene coding region cDNA.

Fig. 5 is the deduced amino acid sequence (SEQ ID NO:5) of the receptor encoded by the nucleotide sequence of Fig. 4. The amino acid sequence encoding human PAR4 contains 385 amino acids. The deduced amino acid sequence begins at nucleotides 3-5 (ATG = Met) and ends at nucleotides 1157-1159 (TGA = stop).

Figs. 6A and 6B show the alignment of the deduced amino acid sequences (SEQ ID NO:2, 6-8) of the mouse PAR4, mouse PAR3, mouse PAR2, and mouse PAR1 genes. To indicate homology, gaps (represented by blank spaces) have been introduced into the five sequences. Exact amino acid matches between at least three of the PARs have been enclosed in a shaded box.

Fig. 7 shows the structure of the mouse PAR genes, revealing a small (250bp) intron separating exon 1, which encodes the signal peptide, from exon 2, which encodes the mature receptor protein (SEQ ID NO:3, 9-11).

Fig. 8 pictorially describes the thrombin cleavage site and activating peptide of the mouse PAR4 receptor.

Fig. 9 is a bar graph showing the calcium response of oocytes expressing mouse PAR4 upon exposure to different agonists. Agonists include thrombin, the predicted activating peptide for PAR4 (GYPGKF), and the predicted activating peptide for PAR1 (SFLLRN).

Fig. 10 is a bar graph showing the calcium response of oocytes expressing human PAR4 upon exposure to different agonists. Agonists include thrombin, the predicted activating peptide for human PAR4 (GYPGQV), the predicted activating peptide for mPAR4 (GYPGKF), and the predicted activating peptide for PAR1 (SFLLRN).

Fig. 11 is a bar graph showing the calcium response of oocytes expressing mouse PAR4 upon exposure to various serine proteases.

Fig. 12 is a bar graph representing the activation of PAR4 in Xenopus laevis oocytes cell types upon exposure to the tethered ligand peptides of the PARs.

Fig. 13 is a graph showing the aggregation of PAR3 knockout mouse platelets in response to mPAR4 activating peptide GYPGKF.

Fig. 14 is a graph showing ATP secretion (top) and aggregation (bottom) of wild-type mouse platelets in response to mPAR4 activating peptide GYPGKF.

Fig. 15 is a graph depicting the activation of human platelets desensitized to the PAR1 activating peptide by the PAR4 predicted activating peptide.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Before the present protease-activated receptor assays and methods of using such are described, it is to be understood that this invention is not limited to the particular DNA sequences, materials, methods, or processes described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a", "and," and "the" include plural referents unless the contexts clearly dictates otherwise. Thus, for example, reference to "a DNA sequence" includes mixtures and large numbers of such sequences, reference to "an assay" includes assays of the same general type, and reference to "the method" includes one or more methods or steps of the type described herein.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications cited herein are incorporated herein by reference for the purpose of disclosing and describing specific aspects of the invention for which the publication is cited in connection with.

DEFINITIONS

By "protease-activated receptor 4", "PAR4", "PAR4 receptor" and the like, is meant all or part of a vertebrate cell surface protein which is specifically activated by thrombin or a thrombin agonist thereby activating PAR4-mediated signaling events (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, platelet aggregation). The polypeptide is characterized as having the

ligand activating properties (including the agonist activating and antagonist inhibiting properties) and tissue distribution described herein. Specifically, PAR4 receptors are expressed by the DNA sequences of SEQ ID NOs: 2, 4, and 5.

By a "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation).

By "substantially pure" is meant that the protease-activated receptor 4 polypeptide provided by the invention is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, PAR4 polypeptide. A substantially pure PAR4 polypeptide may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding a PAR4 polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The protein is substantially pure if it can be isolated to a band in a gel.

By a "substantially identical" amino acid sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the biological activity of the receptor. Such equivalent receptors can be isolated by extraction from the tissues or cells of any animal which naturally produces such a receptor or which can be induced to do so, using the methods described below, or their equivalent; or can be isolated by chemical synthesis; or can be isolated by standard techniques of recombinant DNA technology, e.g., by isolation of cDNA or genomic DNA encoding such a receptor. Substantially identical receptors have the same biological function, e.g. are activated by the same compound.

By "derived from" is meant encoded by the genome of that organism and present on the surface of a subset of that organism's cells.

By "isolated DNA" is meant DNA that is not in its native environment in terms of not being immediately contiguous with (i.e., covalently linked to) the complete coding sequences with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, recombinant DNA which is incorporated into a

vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes any recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

"Isolated DNA" can mean the DNA is in vectors which are preferably capable of directing expression of the protein encoded by the DNA in a vector-containing cell and further includes cells containing such vectors (preferably eukaryotic cells, e.g., CHO cells (ATCC; Cat. No. CCL 61 or COS-7 cells (ATCC; Cat. No. CRL 1651; and the *Xenopus* oocytes of the type described in the above cited reference Vu, T.-K.H. et al. (1991) Cell 64:1057-1068). Preferably, such cells are stably transfected with such isolated DNA.

By "transformed cell" and "transfected cell", "genetically engineered cell", and the like, is meant a cell into which (or into an ancestor of which) has been introduced, by means of genetic engineering, a DNA molecule encoding a PAR4 (or DNA encoding a biologically active fragment or analog, thereof). Such a DNA molecule is "positioned for expression" meaning that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of the PAR4 protein, or fragment or analog, thereof).

By "antibody" is meant an immunoglobulin protein which is capable of binding an antigen. Antibody as used herein is meant to include the entire antibody as well as any antibody fragments (e.g. $F(ab')_2$, Fab', Fab, Fv) capable of binding the epitope, antigen or antigenic fragment of interest.

Antibodies of the invention are immunoreactive or immunospecific for and therefore specifically and selectively bind to a PAR4 protein. Antibodies for PAR4 are preferably immunospecific -- i.e., not substantially cross-reactive with related materials. Although the term "antibody" encompasses all types of antibodies (e.g., monoclonal) the antibodies of the invention are preferably produced using the phage display methodology described herein. The preferred antibody of the invention is a purified antibody. By purified antibody is meant one which is sufficiently free of other proteins, carbohydrates, and lipids with which it is naturally associated. Such an antibody "preferentially binds" to a PAR4 protein (or an antigenic fragment thereof), i.e., does not substantially recognize and bind to other antigenically-unrelated molecules.

By "specifically activates", as used herein, is meant an agent, such as thrombin, a thrombin analog, a PAR4 agonist or other chemical agent including polypeptides such as an antibody, which activates protease-activated receptor 4, receptor polypeptide or a fragment or analog thereof to initiate PAR4-mediated biological events as described herein, but which does not substantially bind other molecules in a sample, e.g., a biological sample, which naturally includes a protease-activated receptor 4 polypeptide.

By "specifically inhibits", as used herein, is meant an agent, such as a thrombin analog, a PAR4 antagonist or other chemical agent including polypeptides such as an antibody, which inhibits activation of protease-activated receptor 4, receptor polypeptide or a fragment or analog thereof, such as by inhibiting thrombin or by blocking activation of PAR4 by thrombin or other PAR4 activator. Preferably, the agent activates or inhibits the biological activity *in vivo* or *in vitro* of the protein to which it binds.

By "biological activity" is meant the ability of the protease-activated receptor 4 to bind thrombin or a PAR4 agonist and signal the appropriate cascade of biological events (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation, and the like, as described herein.

By "substantial increase" is meant an increase in activity or other measurable phenotypic characteristic that is at least approximately a 2-fold increase over control level (where control assays are performed in the absence of activator), preferably at least approximately a 5-fold increase, more preferably at least approximately a 10-fold increase in activity over a control assay.

By "substantial decrease" or "substantial reduction" is meant a decrease or reduction in activity or other measurable phenotypic characteristic that is approximately 80% or the control level, preferably reduced to approximately 50% of the control level, or more preferably reduced to approximately 10% or less of the control level.

The terms "screening method" and "assay method" are used to describe a method of screening a candidate compound for its ability to act as an agonist or antagonist of a PAR4 ligand. The method involves: a) contacting a candidate agonist compound with a recombinant protease-activated receptor 4 (or PAR4 agonist-binding fragment or analog); b) measuring activation of the receptor, the receptor polypeptide or the receptor fragment or analog; and c) identifying agonist compounds as those which interact with the recombinant receptor and trigger or block PAR4 activation. Interaction may be cleavage of the receptor

to unmask an intramolecular receptor activating peptide or by mimicking the intramolecular receptor-activating peptide. A tethered ligand may be more difficult to block than a free agonist. Thus, blocking thrombin is the acid test for an antagonist which will block responses by other thrombin substrates. These terms include assays that examine effects on unoccupied receptors as well as assays that utilize displacement of a ligand from an occupied receptor.

By an "agonist" is meant a molecule which mimics a particular activity, in this case, interacting with a PAR4 ligand in a manner which activates thereby triggering the biological events which normally result from the interaction (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation). Preferably, an agonist initiates a substantial increase in receptor activity relative to control assays in the absence of activator or candidate agonist. An agonist may possess the same, less, or greater activity than a naturally-occurring PAR4 ligand.

The terms "antagonist assay", "antagonist screening" and the like, refer to a method of screening a candidate compound for its ability to antagonize interaction between a naturally-occurring activating ligand or an agonist and the PAR4. The method involves: a) contacting a candidate antagonist compound with a first compound which includes a recombinant PAR4 (or agonist-binding fragment or analog) on the one hand and with a second compound which includes thrombin or a PAR4 agonist on the other hand; b) determining whether the first and second compounds interact or are prevented from interaction by the candidate compound; and c) identifying antagonistic compounds as those which interfere with the interaction of the first compound (PAR4 receptor) to the second compound (PAR4 agonist) and which thereby substantially reduce thrombin or PAR4 agonist-activated biological events (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation).

By an "antagonist" is meant a molecule which blocks activation of a PAR4 receptor. This can be done by inhibiting a particular activity such as the ability of thrombin, for example, to interact with a protease-activated receptor 4 thereby triggering the biological events resulting from such an interaction (e.g., phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet secretion, or platelet aggregation). An antagonist may bind to and thereby block activation of a PAR4 receptor.

The terms "treatment", "treating", "treat" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof

and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particular a human, and includes:

(a) preventing the disease or symptom from occurring in a subject which may be predisposed to the disease or symptom but has not yet been diagnosed as having it;

(b) inhibiting the disease symptom, i.e., arresting its development; or

(c) relieving the disease symptom, i.e., causing regression of the disease.

PREFERRED EMBODIMENTS

In preferred embodiments of both screening methods, the recombinant PAR4 is stably expressed by a vertebrate cell which normally presents substantially no PAR4 on its surface (i.e., a cell which does not exhibit any significant thrombin-mediated phosphoinositide hydrolysis or Ca^{2+} efflux in the presence of a PAR activator); the vertebrate cell is a mammalian cell, is a Rat 1 cell, or a COS 7 cell; and the candidate antagonist or candidate agonist is a thrombin analog, PAR4 peptide fragment or analog or other chemical agent including a polypeptide such as an antibody.

The receptor proteins of the invention are likely involved in the activation of vertebrate platelet, leukocyte, and mesenchymal cells in response to wounding, as well as mediating signaling in embryonic development. Such proteins and in particular PAR4 antagonists are useful therapeutics for the treatment of such conditions as thrombosis, atherosclerosis, restenosis, and inflammation associated with normal wound healing and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and glomerulosclerosis. Preferred therapeutics include 1) agonists, e.g., thrombin analogs, PAR4 peptide fragments or analogs thereof, or other compounds which mimic the action of thrombin upon interaction with the protease-activated receptor 4 or mimic the action of an intramolecular receptor activating peptide; and 2) antagonists, e.g., thrombin analogs, antibodies, or other compounds, which block thrombin or protease-activated receptor 4 function by interfering with the thrombin:receptor interaction or by interfering with the receptor intramolecular activating peptide. The dosage would be expected to be comparable with current antiflammatory drugs and should be adjusted based on the age, sex, weight and condition of the patient beginning with small doses and increasing gradually based on responsiveness and toxicity.

Because the receptor component may now be produced by recombinant techniques and because candidate agonists and antagonists may be screened using transformed, cultured cells, the instant invention provides a simple and rapid approach to the identification of useful therapeutics. Isolation of the PAR4 gene (as cDNA or genomic DNA) allows its expression in a cell type which does not normally bear PAR4 on its surface, providing a system for assaying a thrombin:receptor interaction and receptor activation.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make receptor proteins and sequences encoding such proteins and carry out the methodology for finding such DNA sequences and proteins, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts or parts by weight, molecular weight is weight average molecular weight; temperature is in degrees centigrade; and pressure is at or near atmospheric.

There now follows a description of the cloning and characterization of the cDNA, genomic DNA and the receptor protein of the protease-activated receptor 4 from mouse and human. Expression vectors containing and capable of expressing the PAR4 DNA, as well as transformed cells containing and expressing the DNA of the invention are also described. Also described are possible PAR4 agonists and antagonists as well as screening assays for receptor agonists and receptor antagonists.

EXAMPLE 1: ISOLATION OF THE MOUSE AND HUMAN PROTEASE-ACTIVATED RECEPTOR 4

The public expressed sequence tag (EST) database was searched for potential protease-activated receptor sequences by identifying sequences with homology to PAR1, PAR2 and PAR3. One particular EST, clone 400689, was identified in a database search using PAR2 sequences, showing similarity over an eleven amino acid stretch. The EST was further characterized.

The EST sequence was used to obtain mouse and human cDNA and genomic clones by a combination of PCR and hybridization techniques (see, for example, Sambrook, J. et al.

(1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). The nucleotide sequences for the mouse and human PAR4, and deduced amino acid sequences corresponding to these nucleotide sequences, are shown in Figs. 1-4. The mouse PAR4 cDNA contained an open reading frame encoding a 397 amino acid putative G protein-coupled receptor (Fig. 2). The 5' sequence of the PAR4 cDNAs encoded a predicted thrombin cleavage site, suggesting that this new receptor was a novel thrombin receptor. The gene was characterized further as a potential protease-activated receptor.

The predicted protein structure of the mouse PAR4 product showed significant homology in conserved regions when compared to other mouse protease-activated receptors. Homology between the deduced mouse PAR4 amino acid sequence and the amino acid sequences of mouse PAR1, PAR2, and PAR3 are shown by alignment in Figs. 6A and 6B. Amino acids shared by at least three of the receptor molecules are boxed and shaded.

The genomic region containing the mouse PAR4 gene was sequenced, revealing an exon organization characteristic of protease-activated receptors: a first exon encoding the signal peptide region separated from a second exon encoding the majority of the structure of the mature receptor molecule by a characteristic intron of approximately 250 bp (Fig. 7). The mPAR4 thrombin cleavage site is found at the amino terminus of the protein, and the amino acid sequence corresponding to the mPAR4 tethered ligand peptide, or "activating peptide", is directly adjacent to the cleavage site (Fig. 8).

Cloning of mouse PAR4

The mouse PAR4 cDNA used for the functional studies presented below was cloned from a mouse embryo day 14-15 library. The sequence of EST clone 400689 was used to generate two reverse primers, CCAGTCACAGAAGTGTAGAGGAGCAAATGG (R2) and CAAGCCAGACCCCCTTCCCAC (R3), for 5'RACE using cDNA from mouse embryo day 14-15 (Marathon cDNA, Clonetech). The forward primers used were Clonetech AP1 and AP2. Two "nested" PCR reactions were performed, the first using primers AP1 and R2, the second with AP2 and R3. The first PCR reaction conditions were as follows: 95°C for 5 seconds, 72°C for 4 minutes for the first five cycles, and 95°C for 5 seconds, 70°C for 4 minutes for the next five cycles, and 95°C for 2 seconds, 68°C for 4 minutes for the following 25 cycles. The second PCR reaction conditions were 95°C for 1 minute, followed by 95°C for 30 seconds, 68°C for 3 minutes for 25 cycles. A dominant band of 950 bp was

seen following the nested (AP2-R3) reaction. This product was sequenced, providing mPAR4 sequence 5' to the first transmembrane domain.

Sequence to the start codon was obtained with a second series of 5'RACE reactions again using the Clonetech Marathon cDNA and AP1 and AP2 primers. The primary reaction used AP1 and reverse primer CCACAGCCACCACAAGCCCATAGAG (RACE 1) and the second reaction used AP2 and CCCCAGCAAGCAGTGCTTGAGAGCTG (RACE 2). The reaction conditions for AP1-RACE1 were: 95°C for 30 seconds, 68°C for 3 minutes for 25 cycles. The conditions for the nested AP2-RACE 2 reaction were the same except the PCR went for 30 cycles. A dominant 300 bp band was obtained and sequenced.

The EST clone 400689 was sequenced from both ends to confirm the sequence in the EST database. This sequence, however, differed from sequence which was later obtained both from a cDNA obtained by hybridization screening of a bEND (mouse brain endothelial cell line) library and from a BAC genomic clone (Genome Systems). We believe the latter sequence to be correct.

The final functional clone was obtained by generating a PCR product from the start codon to the stop codon indicated by the EST clone using bEND cDNA as a template. The 3' end of this clone was subsequently replaced by subcloning with a genomic fragment at the Nco I site. Both the original product and the clone containing the 3' genomic fragment were tested in oocytes and found to be equally active.

Human PAR4

The human PAR4 sequence was determined using a degenerate PCR scheme. Human PAR4 sequence was obtained using degenerate PCR primers to amplify a 900 bp dominant product from total human genomic DNA. The primers used for the PCR reactions were: TA(A/G)TA(A/G)TA(A/G/T)AT(A/G)AAIGG(G/A)TCIAC(G/A)CA (designated DR1), GTIGGIA(C/T)TICCIGCIAA(C/T)GG(A/C/G/T)(C/T)T (designated DF1), where "I" designates Inosine. Reaction conditions were: 95°C for 4 minutes, followed by 95°C for 1 minute, 50°C for 2.5minutes, 72°C for 1.5 minutes for 50 cycles. Sequencing of the 900 bp product revealed a novel amino acid sequence that was 88% identical to mouse PAR4.

Human megakaryocytic cell lines were screened for PAR4 expression by Northern using the above 900 bp product as a probe and the K562 erythroleukemia cell line found to be positive. 5'RACE was then performed using the GIBCO 5'RACE kit and K562 mRNA per

the manufacturer's instructions. Nested PCR reactions were performed using two reverse primers: CGAGGTTCATCAGCAGCATGG (GSP2A) and TGCGTGTCCAGCAGGGACAG (GSP2B). Conditions for the first reaction using the GIBCO forward anchor primer and GSP2A were: 95°C for 4 minutes, followed by 95°C for 45 seconds, 56°C for 1 minute, and 72 for 1 minute for 35 cycles. The hemi-nested second reaction was performed with the GIBCO anchor primer and GSP2B as follows: 95°C for 4 minutes, followed by 95°C for 45 seconds, 50°C for 1 minute, and 72°C for 1 minute for 30 cycles. A dominant 350 bp band was observed, subcloned and sequenced, providing sequence to the hPAR4 start codon.

The same kit and template was used for 3'RACE. The forward primers used were: CCTTCTTCGTGCCCAGCAAC (3'GSPA) and GCTGCTGCTGCATTACTCGG (3'GSPB). The GIBCO UAP primer was used as a reverse primer and hemi-nested PCR performed. The first reaction consisted of 95°C for 4 minutes followed by 95°C for 45 seconds, 50°C for 1 minute, and 72°C for 4 minutes for 35 cycles. The second reaction consisted of 95°C for 45 seconds, 50°C for 1 minute, and 72°C for 1 minute for 30 cycles. A dominant 1.6 kb band was observed, subcloned and sequenced, providing sequence to a stop codon.

A functional hPAR4 clone was created by PCR using Vent polymerase with primers from the start codon to 50 bp beyond the stop codon using 25 cycles of PCR. The template was K562 cDNA. The PCR product was sequenced and subcloned into an oocyte expression vector for generating cRNA (pFROGGY). Human PAR4 cRNA was microinjected into Xenopus oocytes to demonstrate function.

EXAMPLE 2: POLYPEPTIDE EXPRESSION

Polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of a PAR3 encoding cDNA fragment (e.g., the cDNAs described above) in a suitable expression vehicle, and expression of the receptor.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant receptor protein. The precise host cell used is not critical to the invention. The receptor may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae* or mammalian cells, e.g., COS-6M, COS-7, NIH/3T3, or Chinese Hamster Ovary cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection,

Rockville, MD). The method of transfection and the choice of expression vehicle will depend on the host system selected. Transformation and mammalian cell transfection methods are described, e.g., in Ausubel et al. (Current Protocols in Molecular Biology, John Wiley & Sons, New York, (1989)); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (Pouwels, P.H. et al., (1985), Supp. 1987).

Particularly preferred expression systems are the *Xenopus* oocyte cells of Vu et al. (Vu et al., Cell (1991) *supra*) and insect cells (SF9-baculovirus) transfected with an expression vector containing and expressing a receptor protein or biologically active fragment thereof. DNA encoding the human or mouse PAR4 or an appropriate receptor fragment or analog (as described above) is inserted into the expression vector in an orientation designed to allow expression. Alternatively, the PAR4 (or biologically active receptor fragment or analog) is expressed by a stably-transfected mammalian cell line. Other preferable host cells which may be used in conjunction with the expression vehicle include NIH/3T3 cells (ATCC Accession No. 1658). The expression may be used in a screening method of the invention (described below) or, if desired, the recombinant receptor protein may be isolated as described below.

A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding the receptor (or receptor fragment or analog) is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the PAR 4-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

One particularly preferred stable expression system is a Rat 1 cell (ATCC) stably transfected with a pcDNAI/NEO (InVitrogen, San Diego, CA) expression vector.

Expression of the recombinant receptor (e.g., produced by any of the expression systems described herein) may be assayed by immunological procedures, such as Western blot or immunoprecipitation analysis of recombinant cell extracts, or by immunofluorescence of intact recombinant cells (using, e.g, the methods described in Ausubel et al., supra). Recombinant receptor protein is detected using an antibody directed to the receptor. Described below are methods for producing anti-protease-activated receptor 4 antibodies using, as an immunogen, the intact receptor or a peptide which includes a suitable protease-activate receptor 4 epitope. To detect expression of a PAR3 fragment or analog, the antibody is preferably produced using, as an immunogen, an epitope included in the fragment or analog.

Once the recombinant PAR4 protein (or fragment or analog, thereof) is expressed, it is isolated, e.g., using immunoaffinity chromatography. In one example, an anti-PAR4 antibody may be attached to a column and used to isolate intact receptor or receptor fragments or analogs. Lysis and fractionation of receptor-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, (1980)).

Receptors of the invention, particularly short receptor fragments, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, (1984) 2nd ed., The Pierce Chemical Co., Rockford, IL).

EXAMPLE 3: ACTIVATION STUDIES OF THE RECOMBINANT PROTEASE-ACTIVATED RECEPTOR 4

PAR4 was demonstrated to be activated by thrombin when expressed in the *Xenopus* oocyte cells of Vu et al. (Vu et al., Cell (1991) *supra*). The ability of PAR4 to mediate signaling by α-thrombin was tested. *Xenopus* oocytes were microinjected with cRNA encoding epitope-tagged mouse PAR4 (mPAR4). Thrombin-triggered ^{45}Ca release was measured as described in Vu et al. (Vu, T.-K. H. et al. (1991) *supra*). The oocytes were microinjected with either 2.5 or 25 ng of the mPAR4 cRNA. A control set of oocytes were

injected with 25 ng of the mouse PAR1 cDNA, WT5. Surface expression of receptors may be confirmed by M1 antibody binding by the method of Ishii, K. et al. (Ishii, K. et al. (1995) J. Biol. Chem. 270:16435-16440; and Ishii, K. et al. (1993) J. Biol. Chem. 268:9780-9786, which references are herein incorporated by reference in their entirety).

Microinjection of *Xenopus* oocytes with mouse PAR4 cRNA conferred thrombin-dependent ^{45}Ca mobilization (Fig. 9) which reflects agonist-triggered phosphoinositide hydrolysis in this system. α-thrombin was able to induce calcium mobilization in both the PAR1 and PAR4 expressing oocytes, even at injection levels of 1nM. mPAR4 expressing cells treated showed approximately a 25-fold increase of Ca release at both concentrations, and this did not vary significantly between the cells expressing higher or lower concentration of mPAR4. In addition, exposure of mPAR4-injected oocytes to mPAR4's activation peptide, GYPGKF (SEQ ID NO:12), induced PAR4-mediated ^{45}Ca mobilization to approximately a 33-fold increase, which is a 2-fold increase over PAR1-mediated mobilization. Exposure to the PAR1 activating peptide, SFLLRN (SEQ ID NO:13), failed to activate PAR4 even at a concentration of 100μM.

Human PAR4 cRNA was also transcribed in vitro from hPAR4 cDNA using standard techniques as described above. Xenopus oocytes were microinjected with 2ng of cRNA/oocyte, and calcium signaling in response to agonist peptides was measured (Fig. 10). 10nM thrombin caused a 30-fold increase in calcium mobilization in oocytes expressing PAR4, consistent with hPAR4 as a functional thrombin receptor. The human PAR4 activating peptide GYPGQV (SEQ ID NO:14) also showed calcium mobilization at 100μM. The mouse activating peptide, GYPGKF, was even more potent than the human activating peptide, whereas the human PAR1 activating peptide showed no significant activity. The negative controls, uninjected cells and cells expressing irrelevant receptors, also showed no activity.

EXAMPLE 4: SPECIFICITY OF THE RECOMBINANT PROTEASE-ACTIVATED RECEPTOR 4

The specificity of activation of PAR4 was examined by the introduction of a number of the arginine/lysine specific serine proteases and PAR4 activating peptides to *Xenopus* oocytes expressing PAR4 (Fig. 11). Various concentrations of the arginine/lysine specific serine proteases plasmin, trypsin, tissue plasminogen activator (APC), Factor VIIa, Factor

Xa, and thrombin were tested. A variant of the PAR4 activating peptide was also tested. The mPAR4 receptor was activated upon treatment with thrombin, its activating peptide GYPGKF, and the relatively less specific serine protease trypsin. No significant mPAR4 activation was seen in response to the other proteases tested, nor with the treatment of the variant activating peptide GAPGKF, which is expected to lack activity.

The specificity of mPAR4, mPAR1, and mPAR2 signaling was also examined. Protease-triggered ^{45}Ca release was measured in *Xenopus* oocytes expressing mouse PAR1, PAR2 or PAR4 stimulated with the activating peptides of each receptor and the proteases thrombin and trypsin (Fig. 12). Each PAR was activated specifically by its respective activating peptide, and not by the activating peptides of the other mPARs. mPAR2 and mPAR3 showed a small level of activation upon treatment with trypsin, but PAR4 displayed a much greater response with a 24-fold increase in ^{45}Ca release. Finally, PAR1 and PAR4 showed a significant response to thrombin, with PAR4 expressing oocytes exhibiting a greater response to thrombin than thrombin receptor mPAR1.

EXAMPLE 5: PAR4 TISSUE EXPRESSION IN MOUSE AND HUMAN

Northern analysis of mouse tissues revealed that PAR4 mRNA was strongest in the mouse spleen cells. The levels of splenic expression of PAR4 are similar to the expression of PAR3 in megakaryocytes, the predicted site of action for PAR3. The role of PAR4 in mouse tissues awaits elucidation, but the finding of PAR4 in spleen is consistent with a role for PAR4 in mediating activation of platelets and other hematopoietic cells by thrombin.

In situ hybridization of mouse tissue reveals the presence of PAR4 mRNA in splenic megakaryocytes, the platelet precursor cells. Control samples in which hybridization is performed with a sense strand probe control are a negative control for all cell types. Northern analysis of mouse tissues for PAR4 mRNA show signal in spleen, with low levels seen in brain, heart, and other tissues. The spleen is a hematopoietic organ in mouse, and both Northern and *in situ* hybridization data suggest that PAR4 is most abundantly expressed in megakaryocytes in the mouse.

The *in situ* hybridization studies are performed as follows. Anesthetized adult C57BL/6 mice are perfusion-fixed with 4% paraformaldehyde. Organs to be tested are dissected, trimmed, and immersion-fixed for 4 hours in 4% paraformaldehyde. Processed tissues are embedded in paraffin, and 5 mm sections were cut. Sense or antisense ^{35}S-

riboprobe are transcribed *in vitro* from mouse PAR2 cDNA subcloned into the *Eco*R1 site of pBluescript II SK$^-$ (Stratagene, San Diego, CA). Hybridization, wash, and development conditions are as reported for mouse PAR1 (Soifer, S.J. et al. (1993) Am. J. Pathol. 144:60-69). To carry out Northern analysis, a ^{32}P-labeled probe for the mouse message is generated by random priming (Prime-It II kit; Stratagene) of PCR-amplified DNA fragments corresponding to mouse cDNA codons representing transmembrane domains 2 to 3. High stringency hybridizations and washes were performed as per the Clonetech protocol for Northern analysis.

EXAMPLE 6: ASSAYS FOR PAR4 FUNCTION

Useful receptor fragments or analogs of the invention are those which interact with thrombin and are activated to initiate the cascade of events associated with thrombin receptor interaction. Such an interaction may be detected by an *in vitro* functional assay method (e.g., the phosphoinositide hydrolysis assay, ^{45}Ca efflux assay, or platelet aggregation assay described herein). This method includes, as components, thrombin and a recombinant protease-activated receptor 4 (or a suitable fragment or analog) configured to permit thrombin binding (e.g., those polypeptides described herein). Thrombin may be obtained from Sigma Chemical Co. (St. Louis, MO) or similar supplier.

Preferably, the protease-activated receptor 4 component is produced by a cell that naturally presents substantially no receptor on its surface, e.g., by engineering such a cell to contain nucleic acid encoding the receptor component in an appropriate expression system. Suitable cells are, e.g., those discussed above with respect to the production of recombinant receptor, such as Rat 1 cells or COS-7 cells.

EXAMPLE 7: SCREENING FOR PROTEASE-ACTIVATED RECEPTOR 4 ACTIVATOR ANTAGONISTS AND AGONISTS

Antagonists

As discussed above, one aspect of the invention features screening for compounds that inhibit the interaction between thrombin (or other PAR4 activating compound) and the protease-activated receptor 4, thereby preventing or reducing the cascade of events that are mediated by that interaction. The elements of the screen are a PAR4 activator (such as thrombin), a candidate antagonist, and recombinant PAR4 (or a suitable receptor fragment or

analog, as outlined above) configured to permit detection of PAR4 activator, antagonist, and PAR4 function. An additional element may be ^{45}Ca, Fura-2, ^{3}H-inositol, or another indicator used to detect downstream signaling (Ishii, K. et al. (1993) *supra*; and Nanevicz, T. et al. (1996) *supra*).

Inhibition of thrombin-induced platelet aggregation may also be used as a means of monitoring an antagonist of PAR4 receptor activation. Thrombin is incubated with the candidate inhibitory compound (such as a peptide) for 5 minutes, then the mixture is added to washed platelets and platelet activation is followed as platelet ATP secretion by lumiaggregometry (see, for example, Connolly, A.J. et al. Nature 381:516-519 (1996); and USPN 5,256,766). Alternately, platelets are incubated with a candidate PAR 4 antagonist for 5 minutes. Thereafter the response to thrombin is measured.

Inclusion of potential antagonists in the screening assay along with thrombin allows for the screening and identification of authentic receptor antagonists as those which decrease thrombin-mediated events, such as platelet aggregation.

Appropriate candidate thrombin antagonists include PAR4 fragments, particularly, fragments of the protein predicted to be extracellular and therefore likely to bind thrombin or the tethered ligand; such fragments would preferably include five or more amino acids. Candidate PAR 4 antagonists include thrombin analogs as well as other peptide and non-peptide compounds and anti-PAR4 antibodies.

Agonists

Another aspect of the invention features screening for compounds that act as PAR4 agonists. Activation of the PAR4 with thrombin or an agonist leads to a cascade of events (such as phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation), providing a convenient means for measuring thrombin or other agonist activity.

The agonist screening assay of the invention utilizes recombinant cells expressing recombinant PAR4 (or a suitable receptor fragment or analog, as outlined herein) configured to permit detection of PAR4 function. Alternatively, a cell such as a leukocyte, a platelet, or a mesenchymal cell that naturally expresses PAR4 may be used. Other elements of the screen include a detectable downstream substrate of the PAR4 activation, such as radiolabelled phosphoinositide, the hydrolysis of which to a detectable product indicates PAR4 activation by the candidate agonist.

^{45}Ca efflux from a cell expressing PAR4 may be used as a means of measuring receptor activation by candidate agonists (Williams, J.A. et al., (1988) PNAS USA 85:4939-4943; Vu, T.-K. H., et al. (1991) Cell 64:1057-1068; and USPN 5,256,766, which references are herein incorporated by reference in their entirety). ^{45}Ca release by oocytes expressing cRNA encoding PAR4 are assessed as follows. Briefly, intracellular calcium pools are labeled by incubating groups of 30 oocytes in 300 µl calcium-free MBSH containing 50 µCi $^{45}CaCl_2$ (10-40 mCi/mg Ca; Amersham) for 4 hours at room temperature. The labeled oocytes are washed, then incubated in MBSH II without antibiotics for 90 minutes. Groups of 5 oocytes are selected and placed in individual wells in a 24-well tissue culture plate (Falcon 3047) containing 0.5 ml/well MBSH II without antibiotics. This medium is removed and replaced with fresh medium every 10 minutes, the harvested medium is analyzed by scintillation counting to determine ^{45}Ca released by the oocytes during each 10-minute incubation. The 10-minute incubations are continued until a stable baseline of ^{45}Ca release per unit time is achieved. Two additional 10-minute collections are obtained, then test medium including agonist is added and agonist-induced ^{45}Ca release determined.

A voltage clamp assay provides an alternative method of monitoring agonist activity. Agonist-induced inward chloride currents are measured in voltage-clamped oocytes expressing thrombin receptor encoding cRNA essentially as previously described (Julius, D. et al. Science (1988) 241:558-563, herein incorporated by reference in its entirety) except that either the single electrode voltage-clamp technique or a two electrode technique may be employed. Platelet aggregation may also be used as a means of monitoring PAR4 receptor activation (see, for example, Connolly, A.J. et al. Nature 381:516-519 (1996). Human platelets may use both PAR 1 and PAR 4.

An agonist useful in the invention is one which imitates the normal thrombin-mediated signal transduction pathway leading, e.g., to an increase in phosphoinositide hydrolysis. Appropriate candidate agonists include thrombin analogs or PAR4 tethered ligand domains or other agents which mimic the action of thrombin or the PAR 4 tethered ligand domain. Agonists would be useful for aiding discovery of antagonists.

EXAMPLE 8: ANTI-PROTEASE-ACTIVATED RECEPTOR 4 ANTIBODIES

Protease-activated receptor 4 (or immunogenic receptor fragments or analogs) may be used to raise antibodies useful in the invention. Receptor fragments preferred for the

production of antibodies are those fragments deduced or shown experimentally to be extracellular.

Antibodies directed to PAR4 peptides are produced as follows. Peptides corresponding to all or part of the PAR4 protein are produced using a peptide synthesizer by standard techniques. The peptides are coupled to KLH with m-maleimide benzoic acid N-hydroxysuccinimide ester. The KLH-peptide is mixed with Freund's adjuvant and injected into animals, e.g. guinea pigs or goats, to produce polyclonal antibodies. Monoclonal antibodies may be prepared using the PAR4 polypeptides described above and standard hybridoma technology (see, e.g., Kohler et al., Nature (1975) 256:495, 1975; Kohler et al., Eur. J. Immunol. (1976) 6:292; Kohler et al., Eur. J. Immunol. (1976) 6:511; Hammerling et al., in Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, (1981); and Ausubel et al., supra). Antibodies are purified by peptide antigen affinity chromatography.

Once produced, antibodies are tested for their ability to bind PAR4 by specific binding to the surface of PAR4-transfected cells or by Western blot or immunoprecipitation analysis (such as by the methods described in Ausubel et al., supra).

Antibodies which specifically recognize PAR4 are considered to be likely candidates for useful antagonists; such candidates are further tested for their ability to specifically interfere with the interaction between thrombin and PAR4 (using the functional antagonist assays described herein). Antibodies which antagonize thrombin:PAR4 binding or PAR4 function are considered to be useful antagonists in the invention.

EXAMPLE 9: ACTIVATION OF MOUSE PLATELETS USING THE PAR4 ACTIVATING PEPTIDE

Blood was collected from mice anesthetized with pentobarbitol by cannulating the inferior vena cava at the level of the renal veins. The blood was mixed with 3.15% citrate (15% of total final volume) and spun at 200g for 7 minutes to obtain platelet rich plasma (PRP). EDTA was added to a final concentration of 10mM, and PGE1 added to a final concentration of 1uM. The PRP was spun at 500g for 10 minutes. The platelet pellet was resuspended in platelet buffer (20mM Tris-HCl pH 7.4, 140 mM NaCl, 2.5 mM KCL, 1.0 mM $MgCl_2$, 1 mg.ml glucose, 0.2% BSA) and 1 mM EDTA and 1uM PGE1 in a volume equal to the original PRP volume, and again spun at 500 g for 10 minutes. The second platelet pellet was resuspended in platelet buffer without EDTA or PGE1. The volume was

adjusted such that the OD_{500} was 1.0. After a minimum of 30 minutes on ice, 300 ul of platelet suspensions were used to measure aggregation and secretion in a Chronolog aggregometer according to manufacturer's instructions.

The PAR4 peptide GYPGKF was added to a final concentration of 500 uM. Secretion and aggregation in response to this peptide were measured over 4-10 minutes (Fig. 13). Since mouse platelets also express PAR3, responses to PAR4 peptide were tested in platelets from PAR3 gene knockout mice to exclude the possibility that PAR3 might be mediating responses to the PAR4 peptide. Note persistent responses to PAR4 peptide were noted in these platelets (Fig. 14).

EXAMPLE 10: ACTIVATION OF HUMAN PLATELETS DESENSITIZED TO THE PAR1 ACTIVATING PEPTIDE

To test whether PAR4 might be functionally expressed in human platelets, platelets were prepared by standard techniques and analyzed by lumiaggregometry as above. Because the mouse peptide GYPGKF was a stronger agonist at human PAR4 than the cognate human peptide GYPGQV, GYPGKF was used. Human platelets were activated by GYPGKF. Human platelets also express PAR1 (like PAR3 in mouse platelets). To exclude the possibility that the PAR4 peptide might be cross-reacting with PAR1, we desensitized human platelets with the PAR1 agonist SFLLRN. Note that these platelets failed to respond to a second challenge with SFLLRN, but did respond to GYPGKF (Fig. 15); thus, the PAR4 peptide is not acting through PAR1, strengthening the conclusion that PAR4 is functionally expressed in human platelets.

EXAMPLE 11: THERAPEUTIC USES OF PAR4

Particularly suitable therapeutics for the treatment of wound healing, thrombosis, atherosclerosis, restenosis, inflammation, and other thrombin-mediated signaling disorders are the agonists and antagonists described above formulated in an appropriate buffer such as physiological saline. Where it is particularly desirable to mimic a receptor fragment conformation at the membrane interface, the fragment may include a sufficient number of adjacent transmembrane residues. In this case, the fragment may be associated with an appropriate lipid fraction (e.g., in lipid vesicles or attached to fragments obtained by disrupting a cell membrane). Alternatively, anti-PAR4 antibodies produced as described

above may be used as a therapeutic. Again, the antibodies would be administered in a pharmaceutically-acceptable buffer (e.g., physiological saline). If appropriate, the antibody preparation may be combined with a suitable adjuvant.

Antibodies to PAR 4 are useful antagonists which can be formulated as indicated above. Other therapeutically useful antagonists are peptides derived from PAR4 that bind to and block thrombin and include formulation comprising a pharmaceutically acceptable carrier and one or more of the following:

a) the isolated sequence
PAPRGYPGQVCANDSDTLELPD (SEQ ID NO:15);

b) uncleavable thrombin inhibitor
PAPRPYPGQVCANDSDTLELPD (SEQ ID NO:16), wherein the PAR 4 cleavage site is mutated to block cleavage;

c) uncleavable thrombin inhibitor
PAP(hR)GYPGQVCANDSDTLELPD (SEQ ID NO:17), wherein the PAR 4 cleavage site P1 is mutated to block cleavage, and hR is beta-homoarginine (the extra methylene group is in the main chain);

d) uncleavable thrombin inhibitor
(dF)PRPYPGQVCANDSDTLELPD (SEQ ID NO:18), wherein the good active site binding sequence dFPR is substituted for PNPR and dF is D-Phenylalanine;

e) any of a)-d) above where all or part of the
sequence corresponding to GYPGQVCAN is replaced with spacer sequences such as GGG;

f) variations and combinations of a)-e) which
act as antagonists.

The therapeutic preparation is administered in accordance with the condition to be treated. Ordinarily, it will be administered intravenously, at a dosage, of a duration, and with the appropriate timing to elicit the desired response. Appropriate timing refers to, for example, time relative to wounding, time intervals between therapeutic administrations, and the like, at which administration of therapeutic preparation elicits the desired response. Alternatively, it may be convenient to administer the therapeutic orally, nasally, or topically,

e.g., as a liquid or a spray. The dosages are determined to be an amount of the therapeutic agent delivered to an animal that substantially reduces or alleviates disease symptoms. Treatment may be repeated as necessary for substantial reduction or alleviation of disease symptoms.

PAR4 activator agonists can be used for the treatment of bleeding. Antagonists may be useful in controlling the formation of clots that cause heart attack and stroke, mediating inflammation and the proliferative responses to injury in normal wound healing and a variety of diseases including atherosclerosis, restenosis, pulmonary inflammations (ARDS), glomerulosclerosis, etc.

The methods of the invention may be used to screen therapeutic receptor activator agonists and antagonists for their effectiveness in altering thrombin-mediated biological events, such as phosphoinositide hydrolysis or other cell signaling events by the assays described above. Where a non-human mammal is treated or where a therapeutic for a non-human animal is screened, the PAR4 or receptor fragment or analog or the antibody employed is preferably specific for that species.

OTHER EMBODIMENTS

Polypeptides according to the invention include any protease-activated 4 receptors (as described herein). Such receptors may be derived from any source, but are preferably derived from a vertebrate animal, e.g., a human or mouse. These polypeptides are used, e.g., to screen for antagonists which disrupt, or agonists which mimic, a thrombin:receptor interaction.

Polypeptides of the invention also include any analog or fragment of a PAR4 capable of interacting with thrombin. Such analogs and fragments may also be used to screen for PAR4 ligand antagonists or agonists. In addition, that subset of receptor fragments or analogs which bind thrombin and are, preferably, soluble (or insoluble and formulated in a lipid vesicle) may be used as antagonists to reduce the *in vivo* concentration of endogenous thrombin, either circulating concentration or local concentration. The efficacy of a receptor analog or fragment is dependent upon its ability to interact with thrombin; such an interaction may be readily assayed using PAR4 functional assays (e.g., those described herein).

Specific receptor analogs of interest include full-length or partial receptor proteins including an amino acid sequence which differs only by conservative amino acid substitutions,

for example, substitution of one amino acid for another of the same class (e.g., valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the receptors' ability to signal thrombin-mediated events (e.g., as assayed above).

Specific receptor fragments of interest include any portion of the PAR4 which is capable of interacting with thrombin, for example, all or part of the extracellular domains predicted from the deduced amino acid sequence. Such fragments may be useful as antagonists (as described above), and are also useful as immunogens for producing antibodies which neutralize the activity of PAR4 *in vivo* (e.g., by interfering with the interaction between the receptor and thrombin). The area as illustrated in Fig. 8 is most likely to bind thrombin. For the human PAR4 protein, modification of the (R47/G48) cleavage site, e.g. substitution of proline for G48, will render peptides mimicking this site uncleavable. Such peptides will bind thrombin with high affinity.

Extracellular regions of novel protease-activated receptors may be identified by comparison with related proteins of similar structure (e.g., other members of the G-protein-coupled receptor family); useful regions are those exhibiting homology to the extracellular domains of well-characterized members of the family.

Alternatively, from the primary amino acid sequence, the secondary protein structure and, therefore, the extracellular domain regions may be deduced semi-empirically using a hydrophobicity/hydrophilicity calculation such as the Chou-Fasman method (see, e.g., Chou and Fasman, Ann. Rev. Biochem. (1978) 47:251). Hydrophilic domains, particularly ones surrounded by hydrophobic stretches (e.g., transmembrane domains) present themselves as strong candidates for extracellular domains. Finally, extracellular domains may be identified experimentally using standard enzymatic digest analysis, e.g., tryptic digest analysis.

Candidate fragments (e.g., any extracellular fragment) are tested for interaction with thrombin by the assays described herein (e.g., the assay described above). Such fragments are also tested for their ability to antagonize the interaction between thrombin and its endogenous receptor, such as PAR4, using the assays described herein. Analogs of useful receptor fragments (as described above) may also be produced and tested for efficacy as screening components or antagonists (using the assays described herein); such analogs are also considered to be useful in the invention.

Identification of the receptor(s) that mediate thrombin signaling provides potential targets for the development of drugs that block thrombin's undesirable actions or mimic its desirable activities. Thrombin receptor antagonists may be used for inhibition of platelet-dependent thrombosis in the setting of unstable angina and myocardial infarction or for blocking thrombin's proinflammatory actions on endothelial cells in the setting of vascular injury. Thrombin receptor agonists may be used to promote hemostasis and fibroblast proliferation at wound sites.

Unmasked tethered ligand domain peptides may provide lead structures for the development of PAR4 agonists or antagonists.

The instant invention is shown and described herein in what is considered to be the most practical, and preferred embodiments. It is recognized, however, that departures may be made therefrom, which are within the scope of the invention, and that obvious modifications will occur to one skilled in the art upon reading this disclosure.

CLAIMS

That which is claimed is:

1. Substantially pure DNA encoding a protease-activated receptor 4 (PAR4).

2. The substantially pure DNA of claim 1, wherein the DNA encodes the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:5.

3. The substantially pure DNA of claim 2, wherein the DNA is SEQ ID NO:1, SEQ ID NO:4, or degenerate variants thereof.

4. Substantially pure DNA having 50% or greater sequence identity to the DNA sequence of claim 1, wherein the DNA selectively hybridizes to sequences complementary to the DNA of claim 1 under stringent conditions.

5. Substantially pure DNA complementary to the DNA of claim 1, wherein the DNA has 50% or greater sequence identity to the DNA of claim 1 and the DNA selectively hybridizes to the DNA of claim 1 under stringent conditions.

6. An isolated PAR4 polypeptide.

7. The isolated polypeptide of claim 9 having an amino acid sequence of SEQ ID NO:2 or SEQ ID NO:5.

8. A fragment or analog of a polynucleotide according to Claim 6 or 7.

9. A substantially pure polypeptide having an amino acid sequence wherein said polypeptide is activated by thrombin; and said polypeptide mediates phosphoinositide hydrolysis in a cell expressing said polypeptide on its surface.

10. A substantially pure PAR4 activating peptide.

11. An antibody which selectively binds to the polypeptide of claim 9.

12. A vector comprising the DNA of claims 1-5.

13. A cell comprising the vector of claim 12.

14. An assay device, comprising:

a support surface;

and a cell of claim 13 or membranes derived therefrom.

15. A therapeutic composition, comprising:

a PAR4 ligand agonist and a physiologically-acceptable carrier.

16. A therapeutic composition, comprising:

a PAR4 ligand antagonist; and a physiologically-acceptable carrier.

```
        10         20         30         40         50         60
ATGTGCTGGCCGCTGCTGTATCCTTTGGTGCTGGGGCTCAGCATCAGCCTGGCAGAGGGC

        70         80         90        100        110        120
ATCCAGACCCCCAGCATCTACGATGATGTAGAGAGTACCAGGGGAAGCCATGAAGGCCCT

       130        140        150        160        170        180
CTGGGTCCCACAGTAGAACTCAAGGAGCCGAAGTCCTCAGACAAGCCTAATCCACGAGGC

       190        200        210        220        230        240
TACCCGGGCAAATTCTGTGCCAACGACAGTGACACGCTGGAGCTCCCGGCCAGCTCTCAA

       250        260        270        280        290        300
GCACTGCTGCTGGGGTGGGTATCCACAAGGCTGGTACCTGCCCTCTATGGGCTTGTGGTG

       310        320        330        340        350        360
GCTGTGGGGCTGCCTGCCAATGGGCTGGCGCTGTGGGTGCTGGCCACAAGGGTGCCACGC

       370        380        390        400        410        420
CTGCCATCCACCATTCTGCTCACGAACCTGGCAGTGGCTGATTCGCTGTTGGCCCTGGTG

       430        440        450        460        470        480
CCGCCACCACGACTGGCTTACCACTTGCGTGGCCAGCGCTGGCCATTTGGTGAGGCTGCC

       490        500        510        520        530        540
TGCCGGGTGGCCACAGCTGCCCTCTATGGCCACATGTATGGTTCAGTGTTGCTGCTGGCT

       550        560        570        580        590        600
GCAGTCAGCTTGGACAGATACCTGGCCCTGGTGCATCCTTTGCGGGCCCGTGCACTGCGT

       610        620        630        640        650        660
GGTCAACGCCTCACTACTGGACTCTGTTTGGTGGCCTGGCTCTCTGCAGCCACCCTGGCC

       670        680        690        700        710        720
TTGCCTCTCACTCTGCATCGGCAGACCTTCCGATTAGCTGGCTCCGATCGCATGCTGTGT

       730        740        750        760        770        780
CATGATGCGCTGCCCCTGACTGAGCAGACCTCCCACTGGAGACCGGCCTTCATCTGCCTG

       790        800        810        820        830        840
GCTGTCCTGGGCTGCTTCGTGCCACTGCTGGCCATGGGCCTGTGCTATGGAGCCACCCTT

       850        860        870        880        890        900
CGTGCACTGGCGGCCAATGGCCAGCGCTACAGCCATGCACTCAGACTGACAGCCCTGGTA

       910        920        930        940        950        960
CTGTTCTCGGCAGTGGCCTCTTTCACACCTAGCAATGTGCTGCTGGTGCTGCACTATTCA

       970        980        990       1000       1010       1020
AACCCGAGCCCTGAGGCCTGGGGCAATCTCTATGGAGCCTATGTGCCCAGCCTGGCACTC

      1030       1040       1050       1060       1070       1080
AGCACCCTCAACAGCTGCGTAGACCCTTTCATCTACTACTATGTGTCCCATGAGTTCAGG

      1090       1100       1110       1120       1130       1140
GAGAAGGTACGCGCTATGTTGTGTCGCCAGCCGGAGGCCAGCAGCTCCTCTCAGGCCTCC
```

Fig. 1A

```
      1150        1160        1170        1180        1190        1200
AGGGAGGCTGGAAGCCGAGGGACTGCCATTTGCTCCTCTACACTTCTGTGACTGGTAGCT

      1210        1220        1230        1240        1250        1260
GAGGTGGGAAGGGGGCATTCTGGCTTGACTGGGTCTCCCCTTAAACTACATCCCTCTTGA

      1270        1280        1290        1300        1310        1320
ACCCTCAGGACATGACCTTATTTGGATATGCAGTTGGTGCGACCTTCATTAGTGGAGCTG

      1330        1340        1350        1360
AGGTCCACTGGAAATGCTTTTGTAAAAGGTCTGGGTACTAT
```

Fig. 1B

```
        10         20         30         40         50         60
MCWPLLYPLVLGLSISLAEGIQTPSIYDDVESTRGSHEGPLGPTVELKEPKSSDKPNPRG

        70         80         90        100        110        120
YPGKFCANDSDTLELPASSQALLLGWVSTRLVPALYGLVVAVGLPANGLALWVLATRVPR

       130        140        150        160        170        180
LPSTILLTNLAVADSLLALVPPPRLAYHLRGQRWPFGEAACRVATAALYGHMYGSVLLLA

       190        200        210        220        230        240
AVSLDRYLALVHPLRARALRGQRLTTGLCLVAWLSAATLALPLTLHRQTFRLAGSDRMLC

       250        260        270        280        290        300
HDALPLTEQTSHWRPAFICLAVLGCFVPLLAMGLCYGATLRALAANGQRYSHALRLTALV

       310        320        330        340        350        360
LFSAVASFTPSNVLLVLHYSNPSPEAWGNLYGAYVPSLALSTLNSCVDPFIYYYVSHEFR

       370        380        390
EKVRAMLCRQPEASSSSQASREAGSRGTAICSSTLL*
```

Fig. 2

```
          10         20         30         40         50         60
GATATGTGCTGGCCGCTGCTGTATCCTTTGGTGCTGGGGCTCANCATCANCCTGGCANAG

          70         80         90        100        110        120
GGCNGGNCACGANNCCCAGCATCTACGATGATGTAGANAGTACCAGGGGAANCCATGGTG

         130        140        150        160        170        180
ANTGACTGTNTCCCTTAAAGGGGTGAATCAGAAATGGAGCTANTGNTGAGCAGGNGNACA

         190        200        210        220        230        240
GNNTTTANGTCCCTAAAANCCATGCCTTTTGGGANTGGGTTGTATCCTTCCNTTAANTGA

         250        260        270        280        290        300
NTNNTGGANTGGGGACANTGAGGCACCCACAATGCCTAAGACTTTCAAGGATATTCTCCT

         310        320        330        340        350        360
TCATCNTGTATCCCTAAAGGCAGGGNAGAGCAGTGGNTGACTGATGTCCCCTCTCTCCCA

         370        380        390        400        410        420
CAGAAGGCCCTNTGGGTCCCACAGTAGAACTCAAGGAGCCGAAGTCCTCAGACAAGCCTA

         430        440        450        460        470        480
ATCCCCGAGGGTACCCGGGCAAATTCTGTGCCAANGACAGTGACACGCTGGAGCTCCCGG

         490        500        510        520
CCAGCTCTCAAGCACTGCNTGNTGGGGTGGGTCCCCACGANTTT
```

Fig. 3

```
        10         20         30         40         50         60
TC-ATGTGGGGGCGACTGCTCCTGTGGCCCCTGGTGCTGGGGTTCAGCCTGTCTGGCGGC

        70         80         90        100        110        120
ACCCAGACCCCCAGCGTCTACGACGAGAGCGGGAGCACCGGAGGTGGTGATGACAGCACG

       130        140        150        160        170        180
CCCTCAATCCTGCCTGCCCCCCGCGGCTACCCAGGCCAAGTCTGTGCCAATGACAGTGAC

       190        200        210        220        230        240
ACCCTGGAGCTCCCGGACAGCTCACGGGCACTGCTTCTGGGCTGGGTGCCCACCAGGCTG

       250        260        270        280        290        300
GTGCCCGCCCTCTATGGGCTGGTCCTGGTGGTGGGGCTGCCGGCCAATGGGCTGGCGCTG

       310        320        330        340        350        360
TGGGTGCTGGCCACGCAGGCACCTCGGCTGCCCTCCACCATGCTGCTGATGAACCTCGCG

       370        380        390        400        410        420
ACTGCTGACCTCCTGCTGGCCCTGGCGCTGCCCCCGCGGATCGCCTACCACCTGCGTGGC

       430        440        450        460        470        480
CAGCGCTGGCCCTTCGGGGAGGCCGCCTGCCGCCTGGCCACGGCCGCACTCTATGGTCAC

       490        500        510        520        530        540
ATGTATGGCTCAGTGCTGCTGCTGGCCGCCGTCAGCCTGGATCGCTACCTGGCCCTGGTG

       550        560        570        580        590        600
CACCCGCTGCGGGCCCGCGCCCTGCGTGGCCGGCGCCTGGCCCTTGGACTCTGCATGGCT

       610        620        630        640        650        660
GCTTGGCTCATGGCGGCCGCCCTGGCACTGCCCCTGACACTGCAGCGGCAGACCTTCCGG

       670        680        690        700        710        720
CTGGCGCGCTCCGATCGCGTGCTCTGCCATGACGCGCTGCCCCTGGACGCACAGGCCTCC

       730        740        750        760        770        780
CACTGGCAACCGGCCTTCACCTGCCTGGCGCTGTTGGGCTGTTTCCTGCCCCTGCTGGCC

       790        800        810        820        830        840
ATGCTGCTGTGCTACGGGGCCACCCTGCACACGCTGGCGGCCAGCGGCCGGCGCTACGGC

       850        860        870        880        890        900
CACGCGCTGAGGCTGACCGCAGTGGTGCTGGCCTCCGCCGTGGCCTTCTTCGTGCCCAGC

       910        920        930        940        950        960
AACCTGCTGCTGCTGCTGCATTACTCGGACCCGAGCCCCAGCGCCTGGGGCAACCTCTAT

       970        980        990       1000       1010       1020
GGTGCCTACGTGCCCAGCCTGGCGCTGAGCACCCTCAACAGCTGCGTGGATCCCTTCATC

      1030       1040       1050       1060       1070       1080
TACTACTACGTGTCGGCCGAGTTCAGGGACAAGGTGCGGGCAGGGCTCTTCCAACGGTCG

      1090       1100       1110       1120       1130       1140
CCGGGGGACACCGTGGCCTCCAAGGCCTCTGCGGAAGGGGGCAGCCGGGGCATGGGCACC
```

Fig. 4A

```
      1150       1160       1170       1180       1190       1200
CACTCCTCTTTGCTCCAGTGACACAAAGTGGGGAAGGCTGTACTGGGTCGAACAGGGTCC

      1210       1220       1230       1240       1250       1260
CTTCCCCCACTTCACGTCCTTCCTGGGACCTCAGAATGTGACCTTATTTGGAAATAGGGT

      1270       1280       1290       1300       1310       1320
TGTTACAACTGTCACTAGCAGAGGTCACTTTGGAGAAGGGTGGGCCTTACATCCAGTGTG

      1330       1340       1350       1360       1370       1380
GGTGGTGTCCTCATAAGATAAGGAGAGGCCAGGCCTGGTGGCTCACGCCTGTAATCCCAG

      1390       1400       1410       1420       1430       1440
CACTTTAAGAGGCCAAGGCGGATGGATCACTTGAGCCCAGGAGTTCAACACCAGCCTGAG

      1450       1460       1470       1480       1490       1500
CAACATGGTAAAACCCCATCTCTACCAAAAATACAAAAATTAGCTGGGCTTGGTGGCTGG

      1510       1520       1530
CGCCTGTAATCCCAGCTACTCANGAGACTGAGGCA
```

Fig. 4B

```
        10         20         30         40         50         60
MWGRLLLWPLVLGFSLSGGTQTPSVYDESGSTGGGDDSTPSILPAPRGYPGQVCANDSDT

        70         80         90        100        110        120
LELPDSSRALLLGWVPTRLVPALYGLVLVVGLPANGLALWVLATQAPRLPSTMLLMNLAT

       130        140        150        160        170        180
ADLLLALALPPRIAYHLRGQRWPFGEAACRLATAALYGHMYGSVLLLAAVSLDRYLALVH

       190        200        210        220        230        240
PLRARALRGRRLALGLCMAAWLMAAALALPLTLQRQTFRLARSDRVLCHDALPLDAQASH

       250        260        270        280        290        300
WQPAFTCLALLGCFLPLLAMLLCYGATLHTLAASGRRYGHALRLTAVVLASAVAFFVPSN

       310        320        330        340        350        360
LLLLLHYSDPSPSAWGNLYGAYVPSLALSTLNSCVDPFIYYYVSAEFRDKVRAGLFQRSP

       370        380
GDTVASKASAEGGSRGMGTHSSLLQ*
```

Fig. 5

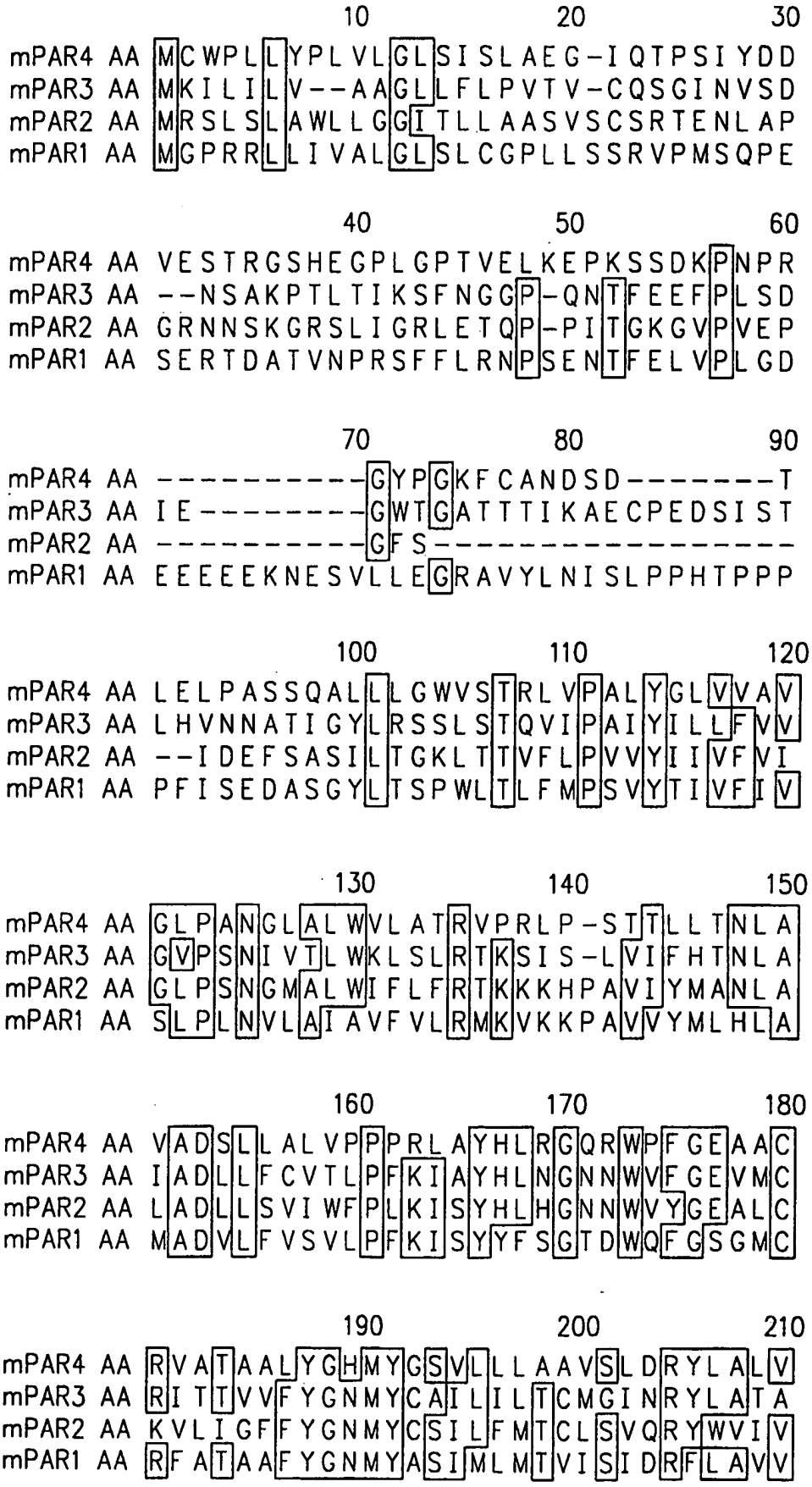
10 20 30
mPAR4 AA MCWPLLYPLVLGLSISLAEG-IQTPSIYDD
mPAR3 AA MKILILV--AAGLLFLPVTV-CQSGINVSD
mPAR2 AA MRSLSLAWLLGGITLLAASVSCSRTENLAP
mPAR1 AA MGPRRLLIVALGLSLCGPLLSSRVPMSQPE
40 50 60
mPAR4 AA VESTRGSHEGPLGPTVELKEPKSSDKPNPR
mPAR3 AA --NSAKPTLTIKSFNGGP-QNTFEEFPLSD
mPAR2 AA GRNNSKGRSLIGRLETQP-PITGKGVPVEP
mPAR1 AA SERTDATVNPRSFFLRNPSENTFELVPLGD
70 80 90
mPAR4 AA ----------GYPGKFCANDSD-------T
mPAR3 AA IE--------GWTGATTTIKAECPEDSIST
mPAR2 AA ----------GFS-----------------
mPAR1 AA EEEEEKNESVLLEGRAVYLNISLPPHTPPP
100 110 120
mPAR4 AA LELPASSQALLLGWVSTRLVPALYGLVVAV
mPAR3 AA LHVNNATIGYLRSSLSTQVIPAIYILLFVV
mPAR2 AA --IDEFSASILTGKLTTVFLPVVYIIVFVI
mPAR1 AA PFISEDASGYLTSPWLTLFMPSVYTIVFIV
130 140 150
mPAR4 AA GLPANGLALWVLATRVPRLP-STTLLTNLA
mPAR3 AA GVPSNIVTLWKLSLRTKSIS-LVIFHTNLA
mPAR2 AA GLPSNGMALWIFLFRTKKKHPAVIYMANLA
mPAR1 AA SLPLNVLAIAVFVLRMKVKKPAVVYMLHLA
160 170 180
mPAR4 AA VADSLLALVPPPRLAYHLRGQRWPFGEAAC
mPAR3 AA IADLLFCVTLPFKIAYHLNGNNWVFGEVMC
mPAR2 AA LADLLSVIWFPLKISYHLHGNNWVYGEALC
mPAR1 AA MADVLFVSVLPFKISYYFSGTDWQFGSGMC
190 200 210
mPAR4 AA RVATAALYGHMYGSVLLLAAVSLDRYLALV
mPAR3 AA RITTVVFYGNMYCAILILTCMGINRYLATA
mPAR2 AA KVLIGFFYGNMYCSILFMTCLSVQRYWVIV
mPAR1 AA RFATAAFYGNMYASIMLMTVISIDRFLAVV

FIG. 6A

```
                          220                 230                 240
mPAR4 AA HPLRARALRGQRLTTGLCLVAWLSAATLAL
mPAR3 AA HPFTYQKLPKRSFSLLMCGIVWVMVFLYML
mPAR2 AA NPMGHPRK-KANIAVGVSLAIWLLIFLVTI
mPAR1 AA YPIQSLSWRTLGRANFTCVVIWVMAIMGVV

                          250                 260                 270
mPAR4 AA PLTLHRQTFRLAGSDRMLCHDALP-LTEQT
mPAR3 AA PFVILKQEYHLVHSEITTCHDVVDACESPS
mPAR2 AA PLYVMKQTIYIPALNITTCHDVLP-EEVLV
mPAR1 AA PLLLKEQTTRVPGLNITTCHDVLS-ENLMQ

                          280                 290                 300
mPAR4 AA SHWRPAFICLAVLGCFVPLLAMGLCYGATL
mPAR3 AA SFRFYYFVSLAFFGFLIPFVIIIFCYTTLI
mPAR2 AA GDMFNYFLSLAIGVFLFPALLTASAYVLMI
mPAR1 AA GFYSYYFSAFSAIFFLVPLIVSTVCYTSII

                          310                 320                 330
mPAR4 AA RALAAN-------GQRYSHALRLTALVLFSA
mPAR3 AA HKLKS-------KDRIWLGYIKAVLLILVIF
mPAR2 AA KTLRSSAMDEHSEKKRQRAIRLIITVLAMY
mPAR1 AA RCLSSSAVAN--RSKKSRALFLSAAVFCIF

                          340                 350                 360
mPAR4 AA VASFTPSNVLLVLHYSNPSPE-AWGNLYGA
mPAR3 AA TICFAPTNIILVIHHANYYYH-NTDSLYFM
mPAR2 AA FICFAPSNLLLVVHYFLIKTQ-RQSHVYAL
mPAR1 AA IVCFGPTNVLLIVHYLFLSDSPGTEAAYFA

                          370                 380                 390
mPAR4 AA YVPSLALSTLNSCVDPFIYYYVSHEFREKV
mPAR3 AA YLIALCLGSLNSCLDPFLYFVMSK-------
mPAR2 AA YLVALCLSTLNSCIDPFVYYFVSKDFRDHA
mPAR1 AA YLLCVCVSSVSCCIDPLIYYYASSECQRHL

                          400                 410                 420
mPAR4 AA R-AMLCRQPEASSSSQASREAGSRGTAICS
mPAR3 AA ----------VVDQLNP
mPAR2 AA RNALLCRSVRTVNRMQISLSSNKFSRKSGS
mPAR1 AA YSILCCKESSDPNSCNSTGQLMPSKMDTCS

                          430
mPAR4 AA STLL
mPAR3 AA
mPAR2 AA YSSSSTSVKTSY
mPAR1 AA SHLNNSIYKKLLA
```

FIG. 6B

MPAR4 Genomic structure vs. known PARs

```
32 33 34 35 36 37 38 39 40 41 42     56 57 58 59 60 61 62 63 64 65 66

AGTACCAGGGGAAGCCATGAAGGCCCTCTGGGT....CCTAATCCACGAGGCTACCCGGGCAAATTCT
AGTACCAGGGGAAGCCATGgtgant
S  T  R  G  S  H  E  G  P  L  G      P  N  P  R  G  Y  P  G  K  F  C
  tcccctctctcccacagAAGGCCCTCTGGGT....CCTAATCCACGAGGCTACCCGGGCAAATTCT

mPAR1: intron 15 AA NH2 of cleavage, 30 from start
mPAR2: 8 AA from cleavage, 31 from start
mPAR3: 7 AA from cleavage, 30 AA from start
MPAR4:  20 AA from putative cleavage, 38 AA from putative start

mPAR1 ↓
CTGTCTTCCCGCGTCCCTATGAGCCAGCCAGgtaagagctgcggg      Exon1-Intron
CTGTCTTCCCGCGTCCCTATGAGCCAGCCAGAATCAGAGAGGACA     cDNA
L  S  S  R  V  P  M  S  Q  P  E  S  E  R  T       amino acids 20-34
           ctcaattttcttctttcagAATCAGAGAGGACA       Intron-Exon2

mPAR2 ↓
TGCAGCCGGACCGAGAACCTTGCACCGGgtgagc                 Exon1-Intron
TGCAGCCGGACCGAGAACCTTGCACCGGGACGCAACAACAGTAAAGGAA  cDNA
C  S  R  T  E  N  L  A  P  G  R  N  N  S  K  G  R  amino acids 22-38
        gctccgctttctttgtacagGACGCAACAACAGTAAAGGAA    Intron-Exon2

mPAR3
                        G  K  P  T  L  T  I  K
          tttcctttcaatacagGCATAAATGTTTTCAGACAACT

Reminder: consensus  SD: (C/A)AG-gt      intron gt is almost invariant
```

Fig. 7

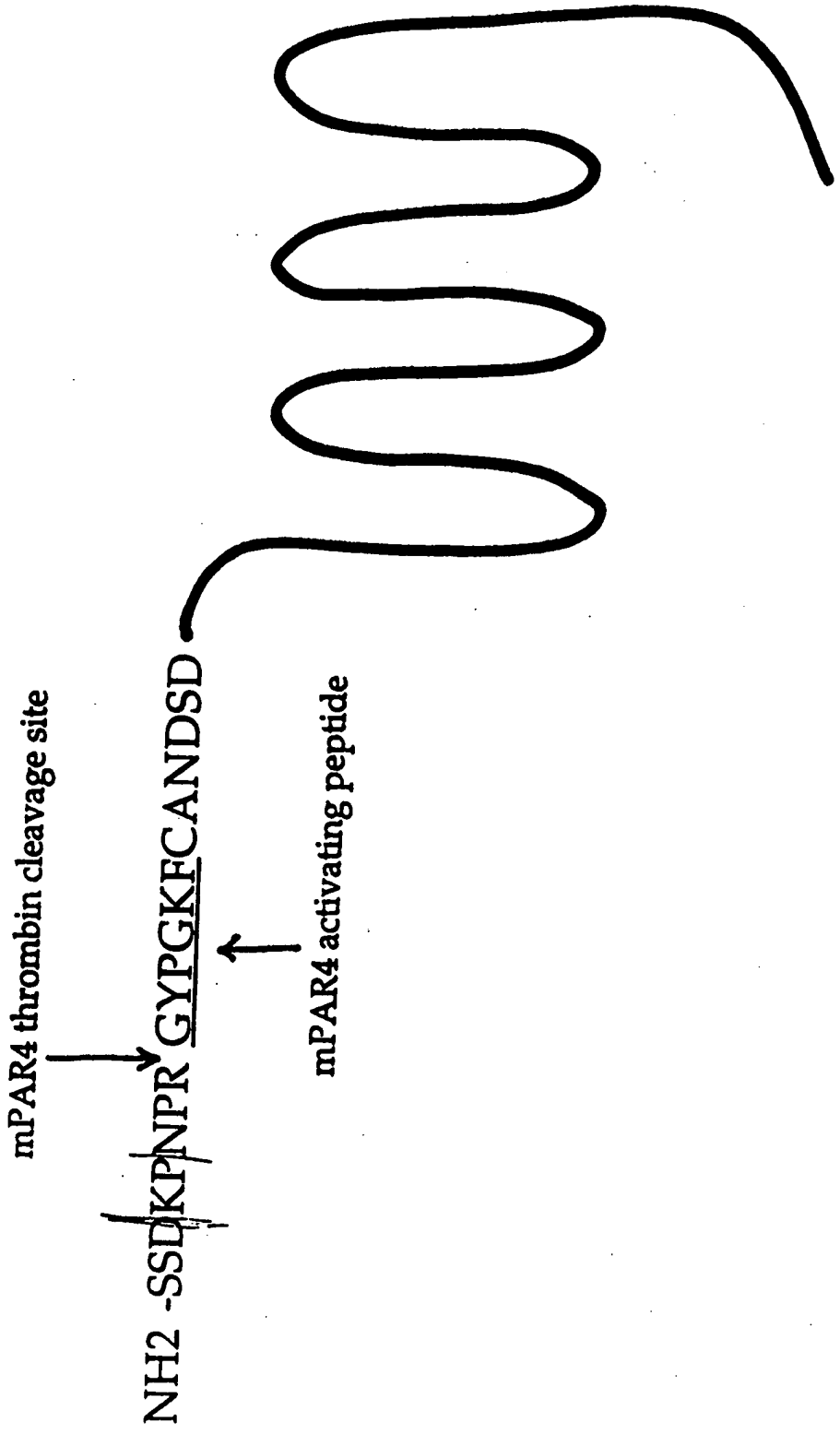
mPAR4 thrombin cleavage site
NH2 -SSDKPNPR GYPGKFCANDSD
mPAR4 activating peptide

Fig. 8

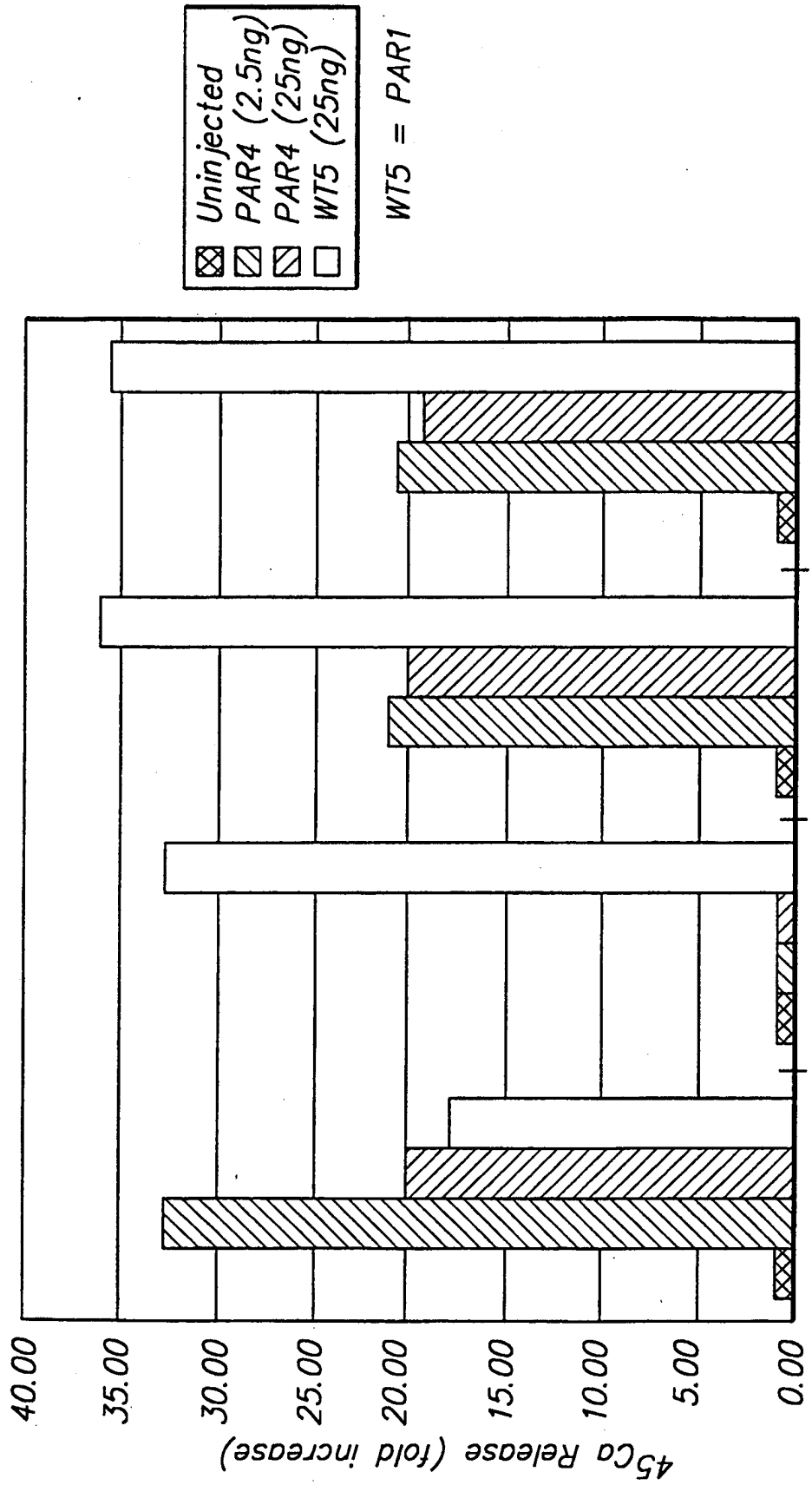
Uninjected
PAR4 (2.5ng)
PAR4 (25ng)
WT5 (25ng)
WT5 = PAR1
45Ca Release (fold increase)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
GYPGKF 10uM
SFLLRN 100uM
aTH 1nM
aTH 10nM
AGONISTS

FIG. 9

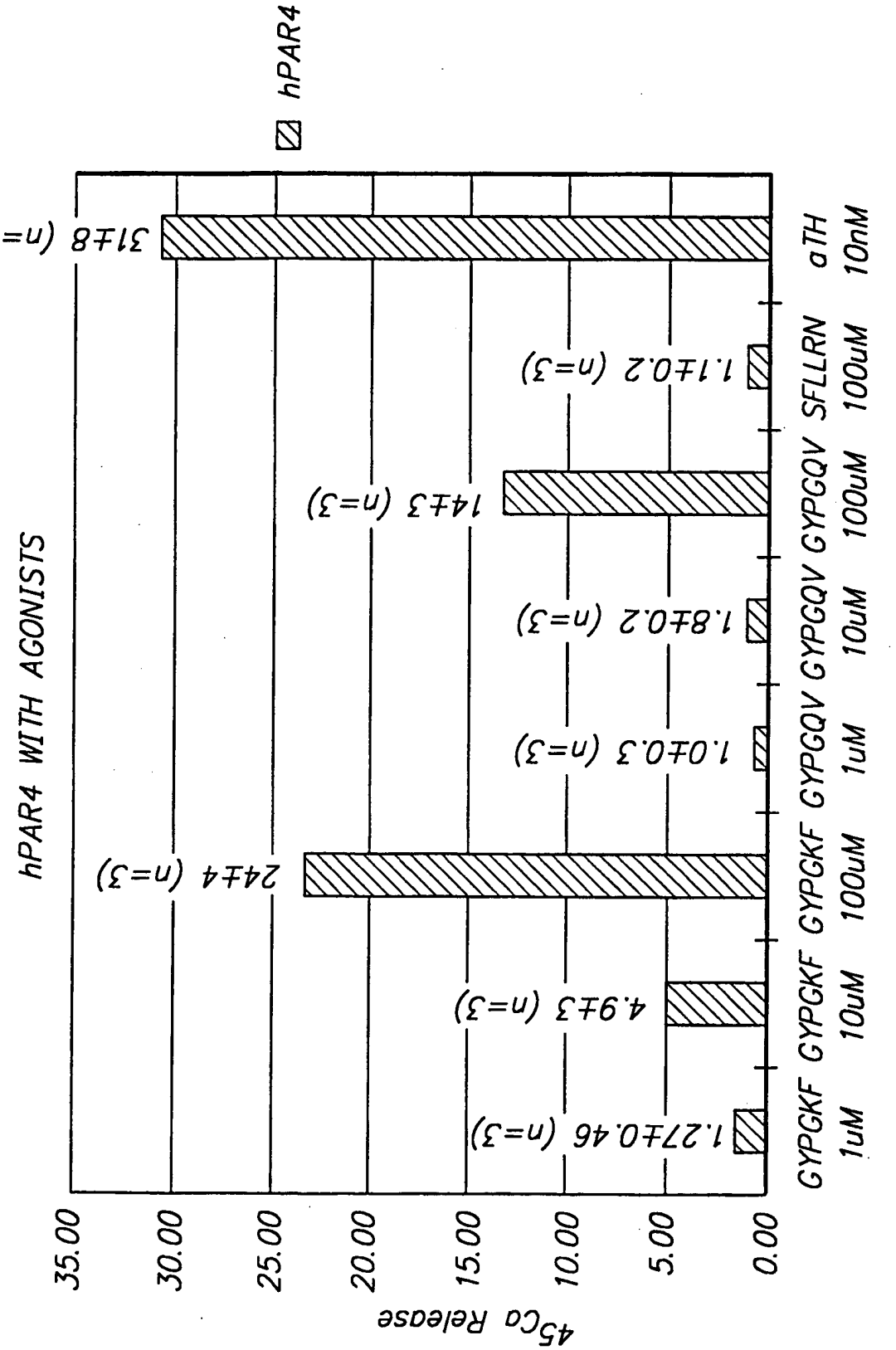
hPAR4
hPAR4 WITH AGONISTS
45Ca Release
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1.27±0.46 (n=3)
4.9±3 (n=3)
24±4 (n=3)
1.0±0.3 (n=3)
1.8±0.2 (n=3)
14±3 (n=3)
1.1±0.2 (n=3)
31±8 (n=3)
GYPGKF GYPGKF GYPGKF GYPGQV GYPGQV GYPGQV SFLLRN aTH
1uM 10uM 100uM 1uM 10uM 100uM 100uM 10nM
AGONISTS

FIG. 10

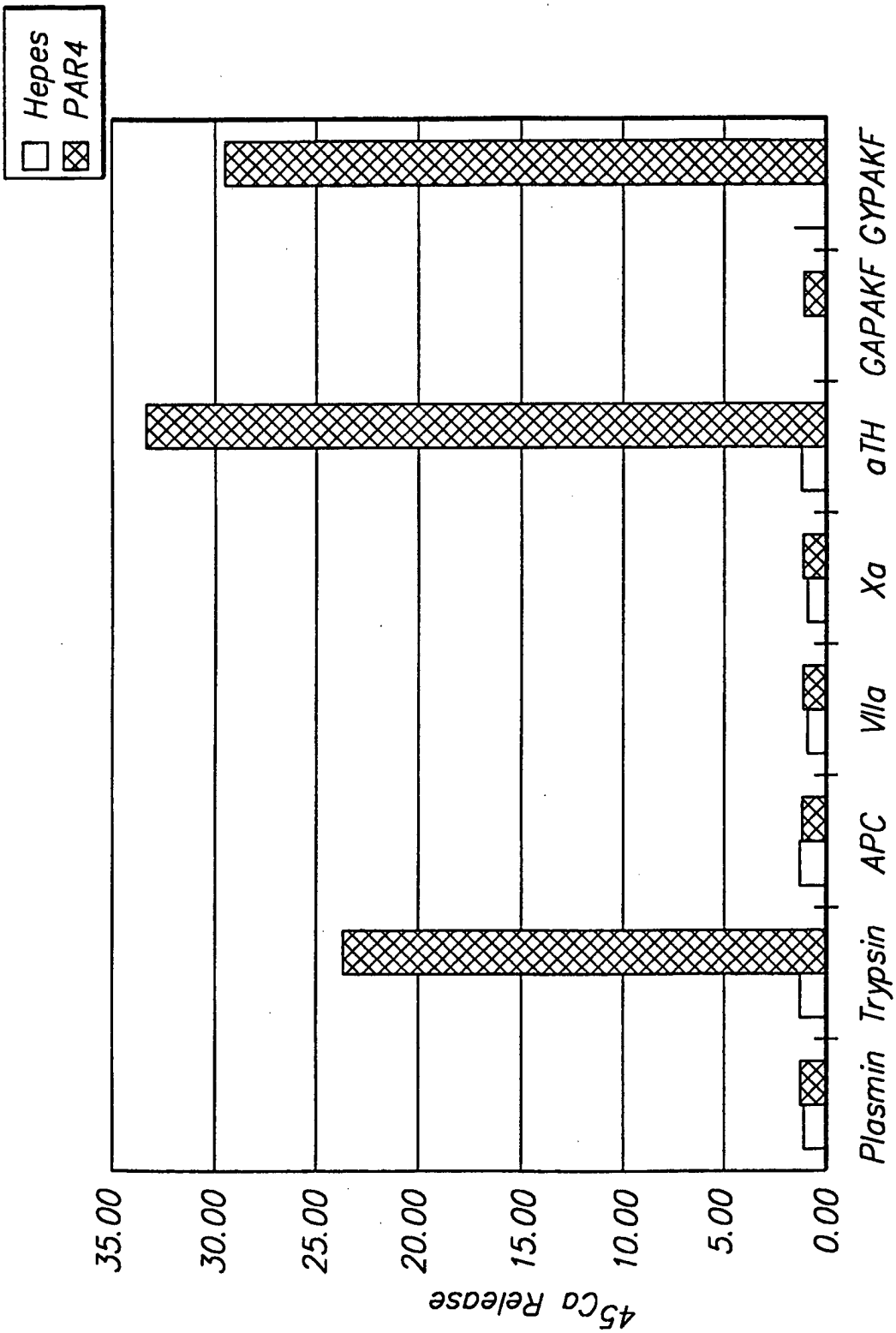
Hepes
PAR4
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
45Ca Release
Plasmin (5nM)
Trypsin (0.5nM)
APC (5nM)
VIIa (5nM)
Xa (5nM)
aTH (5nM)
GAPAKF GYPAKF
AGONISTS

FIG. 11

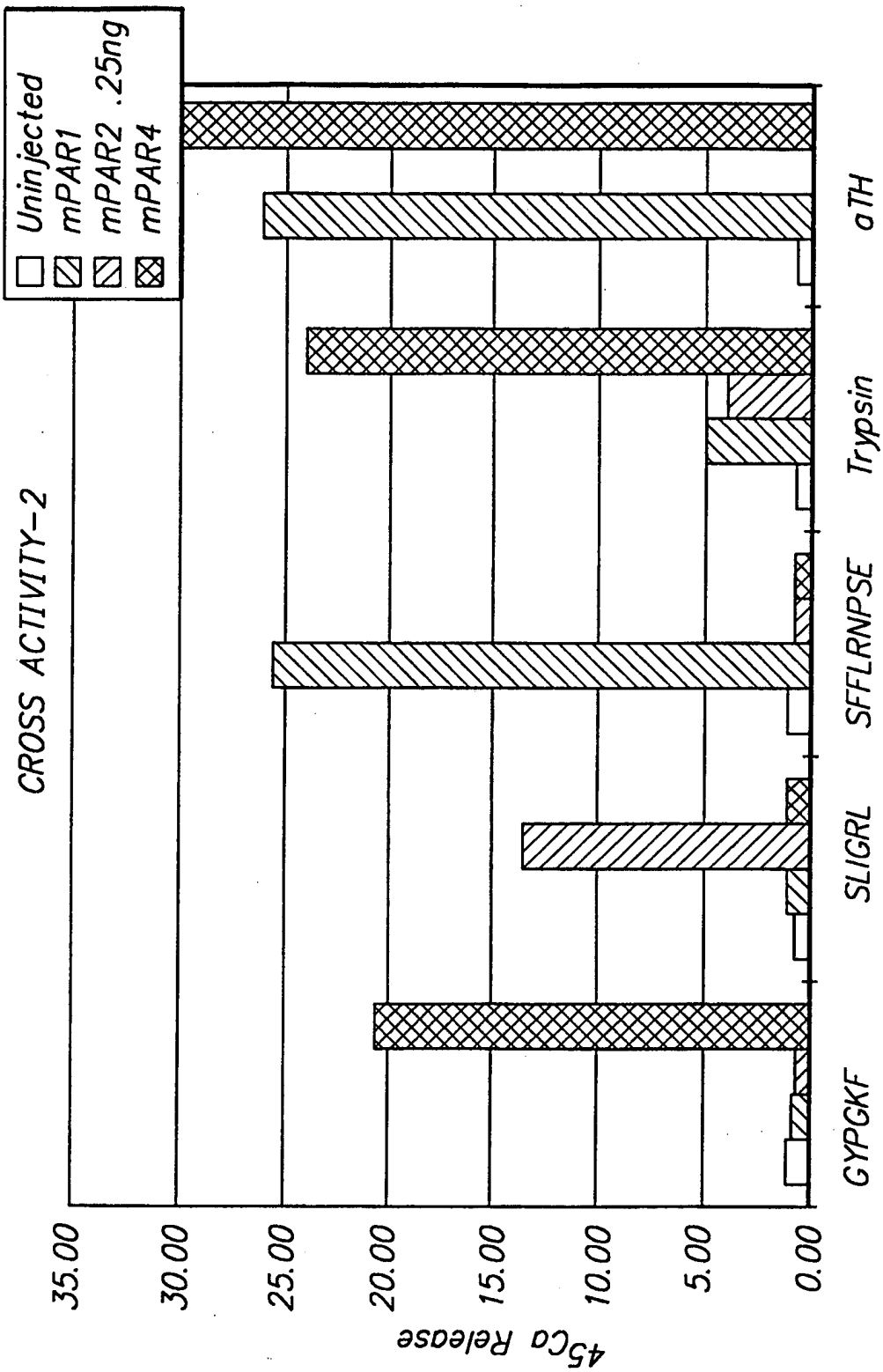
Uninjected
mPAR1
mPAR2 .25ng
mPAR4
CROSS ACTIVITY-2
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
45Ca Release
GYPGKF
SLIGRL
SFFLRNPSE
Trypsin
aTH
AGONISTS

FIG. 12

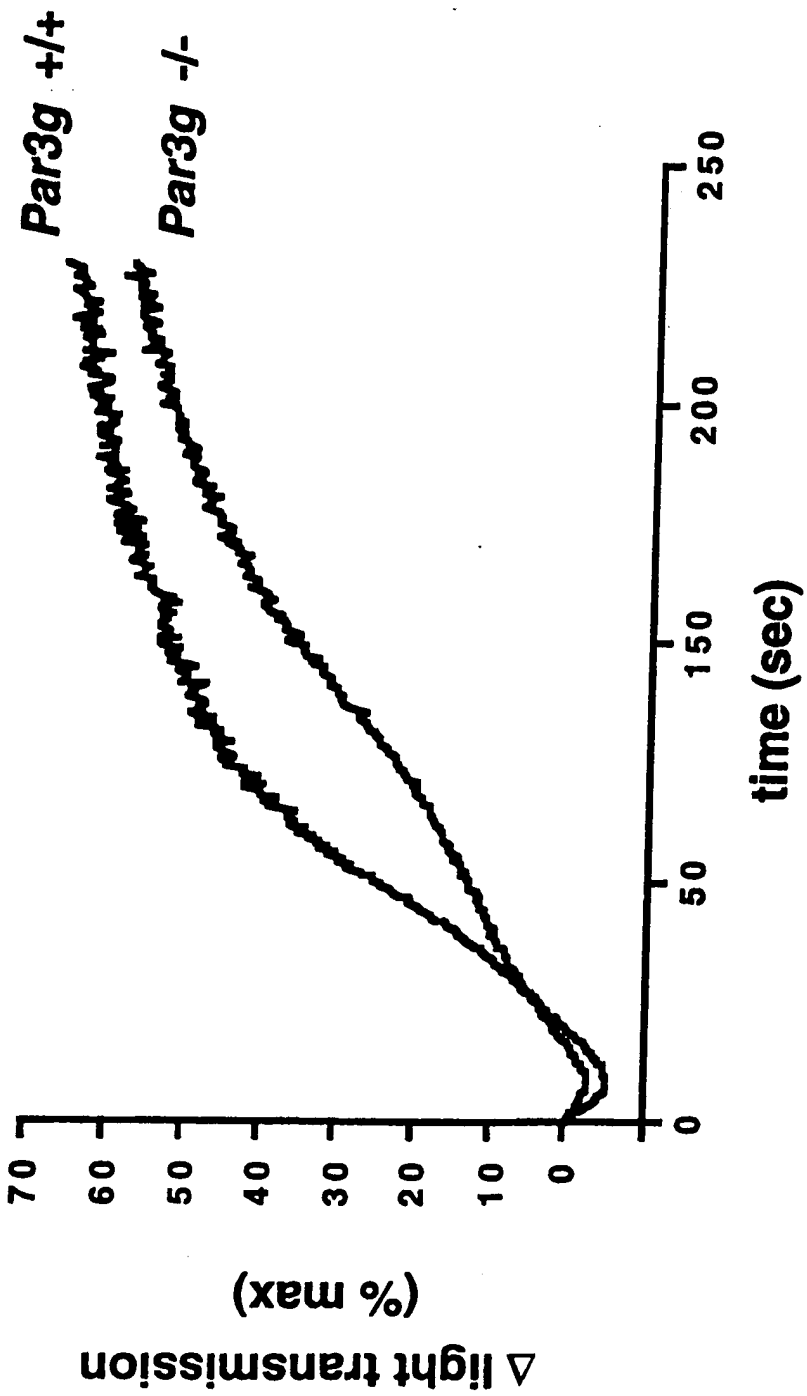
Par3g +/+
Par3g -/-
Δ light transmission
(% max)
70
60
50
40
30
20
10
0
0
50
150
200
250
time (sec)

Fig. 13

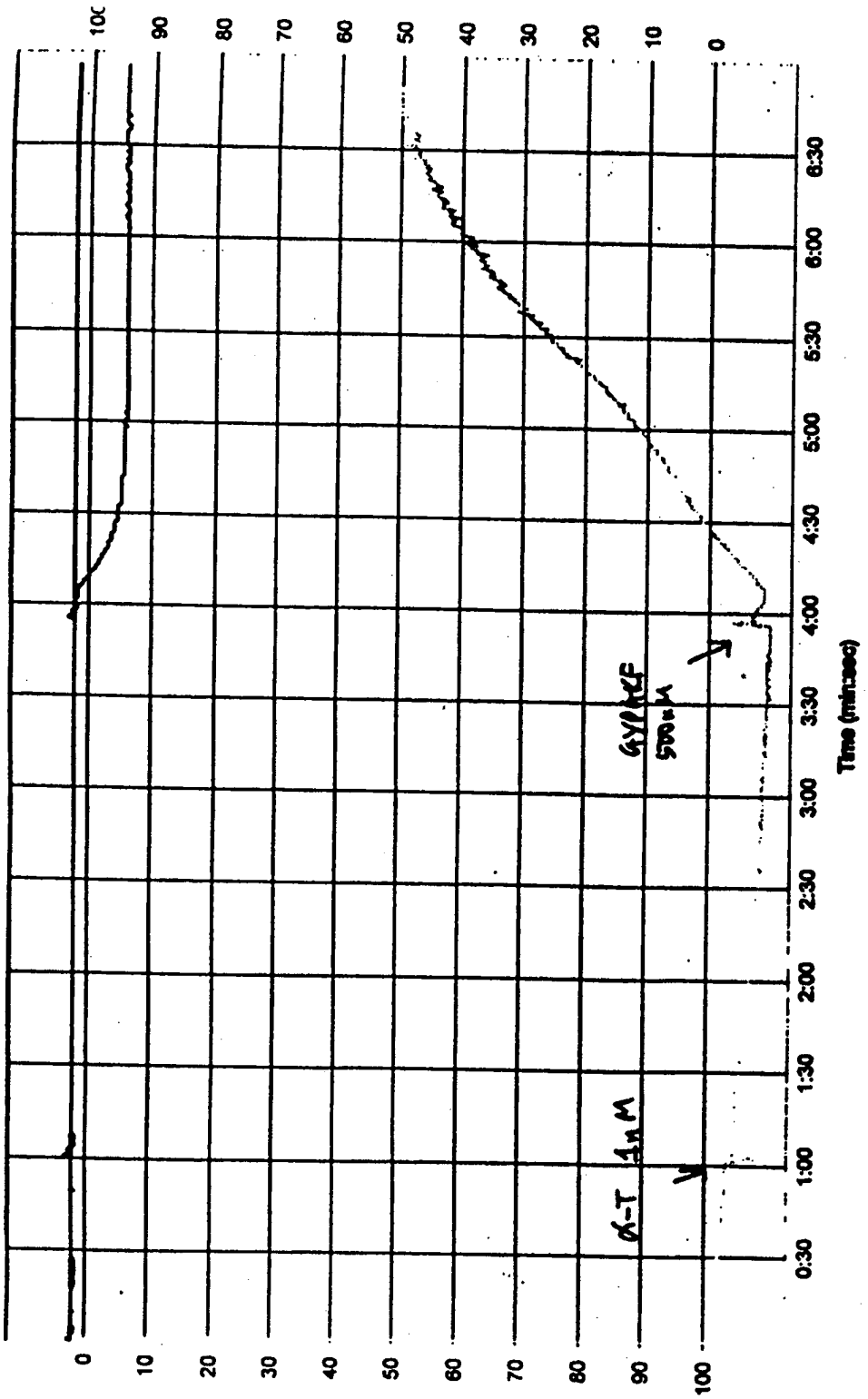
100
90
80
70
60
50
40
30
20
10
0
6:30
6:00
5:30
5:00
4:30
4:00
3:30
3:00
2:30
2:00
1:30
1:00
0:30
Time (minsec)
GYRASE
500nM
α-T 1nM
0
10
20
30
40
50
60
70
80
90
100

Fig. 14

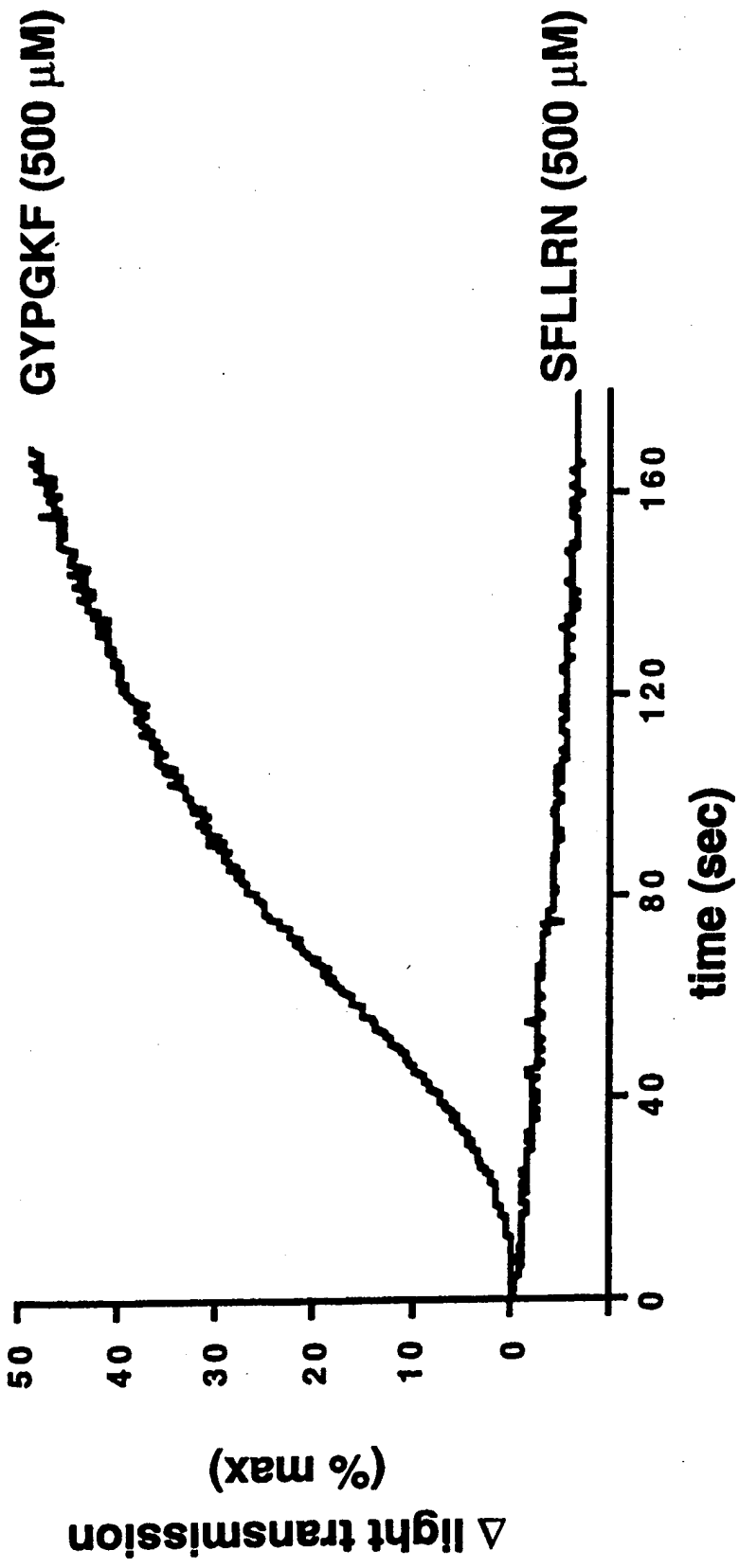
GYPGKF (500 μM)
SFLLRN (500 μM)
Δ light transmission
(% max)
50
40
30
20
10
0
0
40
80
120
160
time (sec)

Fig. 15

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: The Regents of the University of California

(ii) TITLE OF THE INVENTION: Protease Activated Receptor 4 and Uses Thereof

(iii) NUMBER OF SEQUENCES: 30

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Bozicevic, Field & Francis LLP
(B) STREET: 285 Hamilton Avenue, Suite 200
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94301

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: not yet assigned
(B) FILING DATE: herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: USSN 09/032,397
(B) FILING DATE: 27 February 1998 (27/02/98)

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: DeVore, Dianna L
(B) REGISTRATION NUMBER: P-42,484
(C) REFERENCE/DOCKET NUMBER: 06510/093WO1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 650-327-3400
(B) TELEFAX: 650 327-3231
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1360 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGTGCTGGC CGCTGCTGTA TCCTTTGGTG CTGGGGCTCA GCATCAGCCG GCAGAGGGCA 60
TCCAGACCCC CAGCATCTAC GATGATGTAG AGAGTACCAG GGGAAGCCAT GAAGGCCCTC 120
TGGGTCCCAC AGTAGAACTC AAGGAGCCGA AGTCCTCAGA CAAGCCTAAT CCACGAGGCT 180
ACCCGGGCAA ATTCTGTGCC AACGACAGTG ACACGCTGGA GCTCCCGGCC AGCTCTCAAG 240
CACTGCTGCT GGGGTGGGTA TCCACAAGGC TGGTACCTGC CCTCTATGGG CTTGTGGTGG 300
CTGTGGGGCT GCCTGCCAAT GGGCTGGCGC TGTGGGTGCT GGCCACAAGG GTGCCACGCC 360
TGCCATCCAC CATTCTGCTC ACGAACCTGG CAGTGGCTGA TTCGCTGTTG GCCCTGGTGC 420
CGCCACCACG ACTGGCTTAC CACTTGCGTG GCCAGCGCTG GCCATTTGGT GAGGCTGCCT 480
GCCGGGTGGC CACAGCTGCC CTCTATGGCC ACATGTATGG TTCAGTGTTG CTGCTGGCTG 540
CAGTCAGCTT GGACAGATAC CTGGCCCTGG TGCATCCTTT GCGGGCCCGT GCACTGCGTG 600
GTCAACGCCT CACTACTGGA CTCTGTTTGG TGGCCTGGCT CTCTGCAGCC ACCCTGGCCT 660
TGCCTCTCAC TCTGCATCGG CAGACCTTCC GATTAGCTGG CTCCGATCGC ATGCTGTGTC 720
ATGATGCGCT GCCCCTGACT GAGCAGACCT CCCACTGGAG ACCGGCCTTC ATCTGCCTGG 780
CTGTCCTGGG CTGCTTCGTG CCACTGCTGG CCATGGGCCT GTGCTATGGA GCCACCCTTC 840
GTGCACTGGC GGCCAATGGC CAGCGCTACA GCCATGCACT CAGACTGACA GCCCTGGTAC 900
TGTTCTCGGC AGTGGCCTCT TTCACACCTA GCAATGTGCT GCTGGTGCTG CACTATTCAA 960
ACCCGAGCCC TGAGGCCTGG GGCAATCTCT ATGGAGCCTA TGTGCCCAGC CTGGCACTCA 1020
GCACCCTCAA CAGCTGCGTA GACCCTTTCA TCTACTACTA TGTGTCCCAT GAGTTCAGGG 1080
AGAAGGTACG CGCTATGTTG TGTCGCCAGC CGGAGGCCAG CAGCTCCTCT CAGGCCTCCA 1140
GGGAGGCTGG AAGCCGAGGG ACTGCCATTT GCTCCTCTAC ACTTCTGTGA CTGGTAGCTG 1200
AGGTGGGAAG GGGGCATTCT GGCTTGACTG GGTCTCCCCT TAAACTACAT CCCTCTTGAA 1260
CCCTCAGGAC ATGACCTTAT TTGGATATGC AGTTGGTGCG ACCTTCATTA GTGGAGCTGA 1320
GGTCCACTGG AAATGCTTTT GTAAAAGGTC TGGGTACTAT 1360

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 397 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Cys Trp Pro Leu Leu Tyr Pro Leu Val Leu Gly Leu Ser Ile Ser
 1               5                  10                  15
Leu Ala Glu Gly Ile Gln Thr Pro Ser Ile Tyr Asp Asp Val Glu Ser
            20                  25                  30
Thr Arg Gly Ser His Glu Gly Pro Leu Gly Pro Thr Val Glu Leu Lys
        35                  40                  45
Glu Pro Lys Ser Ser Asp Lys Pro Asn Pro Arg Gly Tyr Pro Gly Lys
    50                  55                  60
Phe Cys Ala Asn Asp Ser Asp Thr Leu Glu Leu Pro Ala Ser Ser Gln
65                  70                  75                  80
Ala Leu Leu Leu Gly Trp Val Ser Thr Arg Leu Val Pro Ala Leu Tyr
                85                  90                  95
Gly Leu Val Val Ala Val Gly Leu Pro Ala Asn Gly Leu Ala Leu Trp
            100                 105                 110
Val Leu Ala Thr Arg Val Pro Arg Leu Pro Ser Thr Ile Leu Leu Thr
        115                 120                 125
Asn Leu Ala Val Ala Asp Ser Leu Leu Ala Leu Val Pro Pro Pro Arg
    130                 135                 140
Leu Ala Tyr His Leu Arg Gly Gln Arg Trp Pro Phe Gly Glu Ala Ala
145                 150                 155                 160
Cys Arg Val Ala Thr Ala Ala Leu Tyr Gly His Met Tyr Gly Ser Val
                165                 170                 175
Leu Leu Leu Ala Ala Val Ser Leu Asp Arg Tyr Leu Ala Leu Val His
            180                 185                 190
Pro Leu Arg Ala Arg Ala Leu Arg Gly Gln Arg Leu Thr Thr Gly Leu
        195                 200                 205
Cys Leu Val Ala Trp Leu Ser Ala Ala Thr Leu Ala Leu Pro Leu Thr
    210                 215                 220
Leu His Arg Gln Thr Phe Arg Leu Ala Gly Ser Asp Arg Met Leu Cys
225                 230                 235                 240
His Asp Ala Leu Pro Leu Thr Glu Gln Thr Ser His Trp Arg Pro Ala
                245                 250                 255
Phe Ile Cys Leu Ala Val Leu Gly Cys Phe Val Pro Leu Leu Ala Met
            260                 265                 270
```

```
Gly Leu Cys Tyr Gly Ala Thr Leu Arg Ala Leu Ala Ala Asn Gly Gln
        275                 280                 285
Arg Tyr Ser His Ala Leu Arg Leu Thr Ala Leu Val Leu Phe Ser Ala
    290                 295                 300
Val Ala Ser Phe Thr Pro Ser Asn Val Leu Leu Val Leu His Tyr Ser
305                 310                 315                 320
Asn Pro Ser Pro Glu Ala Trp Gly Asn Leu Tyr Gly Ala Tyr Val Pro
                325                 330                 335
Ser Leu Ala Leu Ser Thr Leu Asn Ser Cys Val Asp Pro Phe Ile Tyr
            340                 345                 350
Tyr Tyr Val Ser His Glu Phe Arg Glu Lys Val Arg Ala Met Leu Cys
        355                 360                 365
Arg Gln Pro Glu Ala Ser Ser Ser Ser Gln Ala Ser Arg Glu Ala Gly
    370                 375                 380
Ser Arg Gly Thr Ala Ile Cys Ser Ser Thr Leu Leu
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 516 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATGTGCTGGC CGCTGCTGTA TCCTTTGGTG CTGGGGCTCA GCATCAGCCG GCAGAGGGCA     60
TCCAGACCCC CAGCATCTAC GATGATGTAG AGAGTACCAG GGGAAGCCAT GGTGANTGAC    120
TGTNTCCCTT AAAGGGGTGA ATCAGAAATG GAGCTANTGN TGAGCAGGNG NACAGNNTTT    180
ANGTCCCTAA AANCCATGCC TTTTGGGANT GGGTTGTATC CTTCCNTTAA NTGANTNNTG    240
GANTGGGGAC ANTGAGGCAC CCACAATGCC TAAGACTTTC AAGGATATTC TCCTTCATCN    300
TGTATCCCTA AAGGCAGGGN AGAGCAGTGG NTGACTGATG TCCCCTCTCT CCCACAGAAG    360
GCCCTCTGGG TCCCACAGTA GAACTCAAGG AGCCGAAGTC CTCAGACAAG CCTAATCCAC    420
GAGGCTACCC GGGCAAATTC TGTGCCAACG ACAGTGACAC GCTGGAGCTC CCGGCCAGCT    480
CTCAAGCACT GCTGCTGGGG TGGGTATCCA CAAGGC                               516
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1534 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCATGTGGGG GCGACTGCTC CTGTGGCCCC TGGTGCTGGG GTTCAGCCTG TCTGGCGGCA 60
CCCAGACCCC CAGCGTCTAC GACGAGAGCG GGAGCACCGG AGGTGGTGAT GACAGCACGC 120
CCTCAATCCT GCCTGCCCCC CGCGGCTACC CAGGCCAAGT CTGTGCCAAT GACAGTGACA 180
CCCTGGAGCT CCCGGACAGC TCACGGGCAC TGCTTCTGGG CTGGGTGCCC ACCAGGCTGG 240
TGCCCGCCCT CTATGGGCTG GTCCTGGTGG TGGGGCTGCC GGCCAATGGG CTGGCGCTGT 300
GGGTGCTGGC CACGCAGGCA CCTCGGCTGC CCTCCACCAT GCTGCTGATG AACCTCGCGA 360
CTGCTGACCT CCTGCTGGCC CTGGCGCTGC CCCCGCGGAT CGCCTACCAC CTGCGTGGCC 420
AGCGCTGGCC CTTCGGGGAG GCCGCCTGCC GCCTGGCCAC GGCCGCACTC TATGGTCACA 480
TGTATGGCTC AGTGCTGCTG CTGGCCGCCG TCAGCCTGGA TCGCTACCTG GCCCTGGTGC 540
ACCCGCTGCG GGCCCGCGCC CTGCGTGGCC GGCGCCTGGC CCTTGGACTC TGCATGGCTG 600
CTTGGCTCAT GGCGGCCGCC CTGGCACTGC CCCTGACACT GCAGCGGCAG ACCTTCCGGC 660
TGGCGCGCTC CGATCGCGTG CTCTGCCATG ACGCGCTGCC CCTGGACGCA CAGGCCTCCC 720
ACTGGCAACC GGCCTTCACC TGCCTGGCGC TGTTGGGCTG TTTCCTGCCC CTGCTGGCCA 780
TGCTGCTGTG CTACGGGGCC ACCCTGCACA CGCTGGCGGC CAGCGGCCGG CGCTACGGCC 840
ACGCGCTGAG GCTGACCGCA GTGGTGCTGG CCTCCGCCGT GGCCTTCTTC GTGCCCAGCA 900
ACCTGCTGCT GCTGCTGCAT TACTCGGACC CGAGCCCCAG CGCCTGGGGC AACCTCTATG 960
GTGCCTACGT GCCCAGCCTG GCGCTGAGCA CCCTCAACAG CTGCGTGGAT CCCTTCATCT 1020
ACTACTACGT GTCGGCCGAG TTCAGGGACA AGGTGCGGGC AGGGCTCTTC CAACGGTCGC 1080
CGGGGGACAC CGTGGCCTCC AAGGCCTCTG CGGAAGGGGG CAGCCGGGGC ATGGGCACCC 1140
ACTCCTCTTT GCTCCAGTGA CACAAAGTGG GGAAGGCTGT ACTGGGTCGA ACAGGGTCCC 1200
TTCCCCCACT TCACGTCCTT CCTGGGACCT CAGAATGTGA CCTTATTTGG AAATAGGGTT 1260
GTTACAACTG TCACTAGCAG AGGTCACTTT GGAGAAGGGT GGGCCTTACA TCCAGTGTGG 1320
GTGGTGTCCT CATAAGATAA GGAGAGGCCA GGCCTGGTGG CTCACGCCTG TAATCCCAGC 1380
ACTTTAAGAG GCCAAGGCGG ATGGATCACT TGAGCCCAGG AGTTCAACAC CAGCCTGAGC 1440
AACATGGTAA AACCCCATCT CTACCAAAAA TACAAAAATT AGCTGGGCTT GGTGGCTGGC 1500
GCCTGTAATC CCAGCTACTC ANGAGACTGA GGCA 1534

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 386 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Trp Gly Arg Leu Leu Leu Trp Pro Leu Val Leu Gly Phe Ser Leu
 1               5                  10                  15
Ser Gly Gly Thr Gln Thr Pro Ser Val Tyr Asp Glu Ser Gly Ser Thr
            20                  25                  30
Gly Gly Gly Asp Asp Ser Thr Pro Ser Ile Leu Pro Ala Pro Arg Gly
        35                  40                  45
Tyr Pro Gly Gln Val Cys Ala Asn Asp Ser Asp Thr Leu Glu Leu Pro
    50                  55                  60
Asp Ser Ser Arg Ala Leu Leu Leu Gly Trp Val Pro Thr Arg Leu Val
65                  70                  75                  80
Pro Ala Leu Tyr Gly Leu Val Leu Val Val Gly Leu Pro Ala Asn Gly
                85                  90                  95
Leu Ala Leu Trp Val Leu Ala Thr Gln Ala Pro Arg Leu Pro Ser Thr
            100                 105                 110
Met Leu Leu Met Asn Leu Ala Thr Ala Asp Leu Leu Leu Ala Leu Ala
        115                 120                 125
Leu Pro Pro Arg Ile Ala Tyr His Leu Arg Gly Gln Arg Trp Pro Phe
    130                 135                 140
Gly Glu Ala Ala Cys Arg Leu Ala Thr Ala Ala Leu Tyr Gly His Met
145                 150                 155                 160
Tyr Gly Ser Val Leu Leu Leu Ala Ala Val Ser Leu Asp Arg Tyr Leu
                165                 170                 175
Ala Leu Val His Pro Leu Arg Ala Arg Ala Leu Arg Gly Arg Arg Leu
            180                 185                 190
Ala Leu Gly Leu Cys Met Ala Ala Trp Leu Met Ala Ala Ala Leu Ala
        195                 200                 205
Leu Pro Leu Thr Leu Gln Arg Gln Thr Phe Arg Leu Ala Arg Ser Asp
    210                 215                 220
Arg Val Leu Cys His Asp Ala Leu Pro Leu Asp Ala Gln Ala Ser His
225                 230                 235                 240
Trp Gln Pro Ala Phe Thr Cys Leu Ala Leu Leu Gly Cys Phe Leu Pro
                245                 250                 255
Leu Leu Ala Met Leu Leu Cys Tyr Gly Ala Thr Leu His Thr Leu Ala
            260                 265                 270
Ala Ser Gly Arg Arg Tyr Gly His Ala Leu Arg Leu Thr Ala Val Val
        275                 280                 285
Leu Ala Ser Ala Val Ala Phe Phe Val Pro Ser Asn Leu Leu Leu Leu
    290                 295                 300
Leu His Tyr Ser Asp Pro Ser Pro Ser Ala Trp Gly Asn Leu Tyr Gly
305                 310                 315                 320
```

```
Ala Tyr Val Pro Ser Leu Ala Leu Ser Thr Leu Asn Ser Cys Val Asp
                325                 330                 335
Pro Phe Ile Tyr Tyr Tyr Val Ser Ala Glu Phe Arg Asp Lys Val Arg
            340                 345                 350
Ala Gly Leu Phe Gln Arg Ser Pro Gly Asp Thr Val Ala Ser Lys Ala
        355                 360                 365
Ser Ala Glu Gly Gly Ser Arg Gly Met Gly Thr His Ser Ser Leu Leu
    370                 375                 380
Gln
385
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 407 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Thr Leu Tyr Thr Xaa Gln His Pro Val Ala Gly Ser Gln Asp Ile Lys
 1               5                  10                  15
Met Lys Ile Leu Ile Leu Val Ala Ala Gly Leu Leu Phe Leu Pro Val
            20                  25                  30
Thr Val Cys Gln Ser Gly Ile Asn Val Ser Asp Asn Ser Ala Lys Pro
        35                  40                  45
Thr Leu Thr Ile Lys Ser Phe Asn Gly Gly Pro Gln Asn Thr Phe Glu
    50                  55                  60
Glu Phe Pro Leu Ser Asp Ile Glu Gly Trp Thr Gly Ala Thr Thr Thr
65                  70                  75                  80
Ile Lys Ala Glu Cys Pro Glu Asp Ser Ile Ser Thr Leu His Val Asn
                85                  90                  95
Asn Ala Thr Ile Gly Tyr Leu Arg Ser Ser Leu Ser Thr Gln Val Ile
            100                 105                 110
Pro Ala Ile Tyr Ile Leu Leu Phe Val Val Gly Val Pro Ser Asn Ile
        115                 120                 125
Val Thr Leu Trp Lys Leu Ser Leu Arg Thr Lys Ser Ile Ser Leu Val
    130                 135                 140
Ile Phe His Thr Asn Leu Ala Ile Ala Asp Leu Leu Phe Cys Val Thr
145                 150                 155                 160
```

```
Leu Pro Phe Lys Ile Ala Tyr His Leu Asn Gly Asn Asn Trp Val Phe
                165                 170                 175
Gly Glu Val Met Cys Arg Ile Thr Thr Val Val Phe Tyr Gly Asn Met
            180                 185                 190
Tyr Cys Ala Ile Leu Ile Leu Thr Cys Met Gly Ile Asn Arg Tyr Leu
        195                 200                 205
Ala Thr Ala His Pro Phe Thr Tyr Gln Lys Leu Pro Lys Arg Ser Phe
    210                 215                 220
Ser Leu Leu Met Cys Gly Ile Val Trp Val Met Val Phe Leu Tyr Met
225                 230                 235                 240
Leu Pro Phe Val Ile Leu Lys Gln Glu Tyr His Leu Val His Ser Glu
                245                 250                 255
Ile Thr Thr Cys His Asp Val Val Asp Ala Cys Glu Ser Pro Ser Ser
            260                 265                 270
Phe Arg Phe Tyr Tyr Phe Val Ser Leu Ala Phe Phe Gly Phe Leu Ile
        275                 280                 285
Pro Phe Val Ile Ile Ile Phe Cys Tyr Thr Thr Leu Ile His Lys Leu
    290                 295                 300
Lys Ser Lys Asp Arg Ile Trp Leu Gly Tyr Ile Lys Ala Val Leu Leu
305                 310                 315                 320
Ile Leu Val Ile Phe Thr Ile Cys Phe Ala Pro Thr Asn Ile Ile Leu
                325                 330                 335
Val Ile His His Ala Asn Tyr Tyr Tyr His Asn Thr Asp Ser Leu Tyr
            340                 345                 350
Phe Met Tyr Leu Ile Ala Leu Cys Leu Gly Ser Leu Asn Ser Cys Leu
        355                 360                 365
Asp Pro Phe Leu Tyr Phe Val Met Ser Lys Val Val Asp Gln Leu Asn
    370                 375                 380
Pro Xaa Ser Ala Met Ala Arg Pro Leu Xaa Arg Pro Arg Arg Asp Ile
385                 390                 395                 400
Trp Glu Asp Ile His Ala Trp
                405
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Arg Ser Leu Ser Leu Ala Trp Leu Leu Gly Gly Ile Thr Leu Leu
 1               5                  10                  15
Ala Ala Ser Val Ser Cys Ser Arg Thr Glu Asn Leu Ala Pro Gly Arg
            20                  25                  30
Asn Asn Ser Lys Gly Arg Ser Leu Ile Gly Arg Leu Glu Thr Gln Pro
        35                  40                  45
Pro Ile Thr Gly Lys Gly Val Pro Val Glu Pro Gly Phe Ser Ile Asp
    50                  55                  60
Glu Phe Ser Ala Ser Ile Leu Thr Gly Lys Leu Thr Thr Val Phe Leu
65                  70                  75                  80
Pro Val Val Tyr Ile Ile Val Phe Val Ile Gly Leu Pro Ser Asn Gly
                85                  90                  95
Met Ala Leu Trp Ile Phe Leu Phe Arg Thr Lys Lys Lys His Pro Ala
            100                 105                 110
Val Ile Tyr Met Ala Asn Leu Ala Leu Ala Asp Leu Leu Ser Val Ile
        115                 120                 125
Trp Phe Pro Leu Lys Ile Ser Tyr His Leu His Gly Asn Asn Trp Val
    130                 135                 140
Tyr Gly Glu Ala Leu Cys Lys Val Leu Ile Gly Phe Phe Tyr Gly Asn
145                 150                 155                 160
Met Tyr Cys Ser Ile Leu Phe Met Thr Cys Leu Ser Val Gln Arg Tyr
                165                 170                 175
Trp Val Ile Val Asn Pro Met Gly His Pro Arg Lys Lys Ala Asn Ile
            180                 185                 190
Ala Val Gly Val Ser Leu Ala Ile Trp Leu Leu Ile Phe Leu Val Thr
        195                 200                 205
Ile Pro Leu Tyr Val Met Lys Gln Thr Ile Tyr Ile Pro Ala Leu Asn
    210                 215                 220
Ile Thr Thr Cys His Asp Val Leu Pro Glu Glu Val Leu Val Gly Asp
225                 230                 235                 240
Met Phe Asn Tyr Phe Leu Ser Leu Ala Ile Gly Val Phe Leu Phe Pro
                245                 250                 255
Ala Leu Leu Thr Ala Ser Ala Tyr Val Leu Met Ile Lys Thr Leu Arg
            260                 265                 270
Ser Ser Ala Met Asp Glu His Ser Glu Lys Lys Arg Gln Arg Ala Ile
        275                 280                 285
Arg Leu Ile Ile Thr Val Leu Ala Met Tyr Phe Ile Cys Phe Ala Pro
    290                 295                 300
Ser Asn Leu Leu Leu Val Val His Tyr Phe Leu Ile Lys Thr Gln Arg
305                 310                 315                 320
```

```
Gln Ser His Val Tyr Ala Leu Tyr Leu Val Ala Leu Cys Leu Ser Thr
                325                 330                 335
Leu Asn Ser Cys Ile Asp Pro Phe Val Tyr Tyr Phe Val Ser Lys Asp
            340                 345                 350
Phe Arg Asp His Ala Arg Asn Ala Leu Leu Cys Arg Ser Val Arg Thr
        355                 360                 365
Val Asn Arg Met Gln Ile Ser Leu Ser Ser Asn Lys Phe Ser Arg Lys
    370                 375                 380
Ser Gly Ser Tyr Ser Ser Ser Ser Thr Ser Val Lys Thr Ser Tyr
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 430 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Gly Pro Arg Arg Leu Leu Ile Val Ala Leu Gly Leu Ser Leu Cys
 1               5                  10                  15
Gly Pro Leu Leu Ser Ser Arg Val Pro Met Ser Gln Pro Glu Ser Glu
            20                  25                  30
Arg Thr Asp Ala Thr Val Asn Pro Arg Ser Phe Phe Leu Arg Asn Pro
        35                  40                  45
Ser Glu Asn Thr Phe Glu Leu Val Pro Leu Gly Asp Glu Glu Glu Glu
    50                  55                  60
Glu Lys Asn Glu Ser Val Leu Leu Glu Gly Arg Ala Val Tyr Leu Asn
65                  70                  75                  80
Ile Ser Leu Pro Pro His Thr Pro Pro Pro Pro Phe Ile Ser Glu Asp
                85                  90                  95
Ala Ser Gly Tyr Leu Thr Ser Pro Trp Leu Thr Leu Phe Met Pro Ser
            100                 105                 110
Val Tyr Thr Ile Val Phe Ile Val Ser Leu Pro Leu Asn Val Leu Ala
        115                 120                 125
Ile Ala Val Phe Val Leu Arg Met Lys Val Lys Lys Pro Ala Val Val
    130                 135                 140
Tyr Met Leu His Leu Ala Met Ala Asp Val Leu Phe Val Ser Val Leu
145                 150                 155                 160
```

```
Pro Phe Lys Ile Ser Tyr Tyr Phe Ser Gly Thr Asp Trp Gln Phe Gly
                165                 170                 175
Ser Gly Met Cys Arg Phe Ala Tyr Ala Ala Phe Tyr Gly Asn Met Tyr
            180                 185                 190
Ala Ser Ile Met Leu Met Thr Val Ile Ser Ile Asp Arg Phe Leu Ala
        195                 200                 205
Val Val Tyr Pro Ile Gln Ser Leu Ser Trp Arg Thr Leu Gly Arg Ala
    210                 215                 220
Asn Phe Thr Cys Val Val Ile Trp Val Met Ala Ile Met Gly Val Val
225                 230                 235                 240
Pro Leu Leu Leu Lys Glu Gln Thr Thr Arg Val Pro Gly Leu Asn Ile
                245                 250                 255
Thr Thr Cys His Asp Val Leu Ser Glu Asn Leu Met Gln Gly Phe Tyr
            260                 265                 270
Ser Tyr Tyr Phe Ser Ala Phe Ser Ala Ile Phe Phe Leu Val Pro Leu
        275                 280                 285
Ile Val Ser Thr Val Cys Tyr Thr Ser Ile Ile Arg Cys Leu Ser Ser
    290                 295                 300
Ser Ala Val Ala Asn Arg Ser Lys Lys Ser Arg Ala Leu Phe Leu Ser
305                 310                 315                 320
Ala Ala Val Phe Cys Ile Phe Ile Val Cys Phe Gly Pro Thr Asn Val
                325                 330                 335
Leu Leu Ile Val His Tyr Leu Phe Leu Ser Asp Ser Pro Gly Thr Glu
            340                 345                 350
Ala Ala Tyr Phe Ala Tyr Leu Leu Cys Val Cys Val Ser Ser Val Ser
        355                 360                 365
Cys Cys Ile Asp Pro Leu Ile Tyr Tyr Tyr Ala Ser Ser Glu Cys Gln
    370                 375                 380
Arg His Leu Tyr Ser Ile Leu Cys Cys Lys Glu Ser Ser Asp Pro Asn
385                 390                 395                 400
Ser Cys Asn Ser Thr Gly Gln Leu Met Pro Ser Lys Met Asp Thr Cys
                405                 410                 415
Ser Ser His Leu Asn Asn Ser Ile Tyr Lys Lys Leu Leu Ala
            420                 425                 430
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 26...67
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGTCTTCCC GCGTCCCTAT GAGCCAGCCA GGTAAGAGCT GCGGGNNNCT CAATTTTCTT 60
CTTTCAGAAT CAGAGAGGAC A 81

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Genomic DNA
(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 29...57
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGCAGCCGGA CCGAGAACCT TGCACCGGGT GAGCNNNGCT CCGCTTTCTT TGTACAGGAC 60
GCAACAACAG TAAAGGAA 78

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Genomic DNA
(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 10...16
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTCCTTTCA ATACAGGCAT AAATGTTTTC AGACAACT 38

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Tyr Pro Gly Lys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser Phe Leu Leu Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Tyr Pro Gly Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Pro Ala Pro Arg Gly Tyr Pro Gly Gln Val Cys Ala Asn Asp Ser Asp
1 5 10 15
Thr Leu Glu Leu Pro Asp
20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Pro Ala Pro Arg Pro Tyr Pro Gly Gln Val Cys Ala Asn Asp Ser Asp
1 5 10 15
Thr Leu Glu Leu Pro Asp
20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 4...0
(D) OTHER INFORMATION: Xaa is hR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Pro Ala Pro Xaa Gly Tyr Pro Gly Gln Val Cys Ala Asn Asp Ser Asp
 1               5                  10                  15
Thr Leu Glu Leu Pro Asp
            20
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 1...0
(D) OTHER INFORMATION: Xaa is dF

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Xaa Pro Arg Pro Tyr Pro Gly Gln Val Cys Ala Asn Asp Ser Asp Thr
 1               5                  10                  15
Leu Glu Leu Pro Asp
            20
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 1...0
(D) OTHER INFORMATION: Xaa is Ala, Ser or Thr

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Xaa Tyr Pro Gly Lys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Phe Pro Gly Lys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 2...0
(D) OTHER INFORMATION: Xaa is parafluoroPhe

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Gly Xaa Pro Gly Lys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Leu Pro Gly Lys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Ile Pro Gly Lys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gly Trp Pro Gly Lys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 1...0
(D) OTHER INFORMATION: Xaa is Ala, Ser or Thr

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Xaa Tyr Pro Gly Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Gly Phe Pro Gly Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 2...0
(D) OTHER INFORMATION: Xaa is parafluoroPhe

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Gly Xaa Pro Gly Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Leu Pro Gly Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Gly Ile Pro Gly Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Gly Trp Pro Gly Gln Val
1 5

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/705, C12N 9/74, C07K 16/28, C12N 1/21, 1/19, 5/10, C12Q 1/00, G01N 33/68, A61K 38/48, 38/17

A3

(11) International Publication Number: WO 99/43809

(43) International Publication Date: 2 September 1999 (02.09.99)

(21) International Application Number: PCT/US99/02983

(22) International Filing Date: 11 February 1999 (11.02.99)

(30) Priority Data:
09/032,397 27 February 1998 (27.02.98) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94607–5200 (US).

(72) Inventors: COUGHLIN, Shaun, R.; 2 Turtle Rock Court, Tiburon, CA 94920 (US). KAHN, Mark; 1337 Carlos Avenue, Burlingame, CA 94010 (US).

(74) Agent: DEVORE, Dianna, L.; Bozicevic, Field & Francis LLP, Suite 200, 285 Hamilton Avenue, Palo Alto, CA 94301 (US).

(81) Designated States: AU, CA, JP, KR, NO, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
14 October 1999 (14.10.99)

(54) Title: PROTEASE–ACTIVATED RECEPTOR 4 AND USES THEREOF

(57) Abstract

Disclosed are cDNAs and genomic DNAs encoding protease–activated receptor 4 (PAR4) from mouse and human, and the recombinant polypeptides expressed from such cDNAs. The recombinant receptor polypeptides, receptor fragments and analogs expressed on the surface of cells are used in methods of screening candidate compounds for their ability to act as agonists or antagonists to the effects of interaction between thrombin and PAR4. Agonists are used as therapeutics to treat wounds, promote clotting, and as reagents to activate platelets in diagnostic tests. Antagonists are used as therapeutics to control blood coagulation, treat heart attack and stroke, and block inflammatory and proliferative responses to injury as occur in normal wound healing and variety of diseases including atherosclerosis, restenosis, pulmonary inflammation (ARDS) and glomerulosclerosis. Antibodies specific for a protease–activated receptor 4 (or receptor fragment or analog) and their use as a therapeutic are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/US 99/02983

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C12N9/74 C07K16/28 C12N1/21
C12N1/19 C12N5/10 C12Q1/00 G01N33/68 A61K38/48
A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 31810 A (SCHERING CORP) 23 July 1998 (1998-07-23) page 66-69; claims page 55, last paragraph - page 57 ---	1-5, 11-14
P,Y	WO 98 18456 A (UNIV CALIFORNIA) 7 May 1998 (1998-05-07) claims 17,18 ---	15,16
A	WO 96 40040 A (INCYTE PHARMA INC). 19 December 1996 (1996-12-19) ---	
A	ISHIHARA, HIROAKI ET AL: "Protease - activated receptor 3 is a second thrombin receptor in humans." NATURE (LONDON), (1997) VOL. 386, NO. 6624, PP. 502-506. , XP002112285 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 1999	30/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

Inter. nal Application No
PCT/US 99/02983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	XU W F ET AL: "Cloning and characterization of human protease - activated receptor 4." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 JUN 9) 95 (12) 6642-6. , XP002112286	1-14
Y	the whole document ---	15,16
P,X	KAHN M L ET AL: "A dual thrombin receptor system for platelet activation." NATURE, (1998 AUG 13) 394 (6694) 690-4. , XP002112287	1-15
	the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/02983

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9831810 A	23-07-1998	AU 6017098 A	07-08-1998
WO 9818456 A	07-05-1998	US 5892014 A	06-04-1999
WO 9640040 A	19-12-1996	US 5686597 A	11-11-1997
		AU 5985896 A	30-12-1996
		CA 2223077 A	19-12-1996
		EP 0832128 A	01-04-1998
		US 5869633 A	09-02-1999